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RUBELLA VIRUS INFECTION OF PRIMARY VILLOUS TROPHOBLASTS

By

KRISTA R. PENNEY

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Master of Science**

in

Virology.

Department of Medical Microbiology and Immunology

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Sandra T. Davidge

May 13, 2003
Date

ABSTRACT

Rubella virus is a major teratogen causing Congenital Rubella Syndrome (CRS), a characteristic set of birth defects. Very little is known about how Rubella virus transmits from the mother to the fetus across the placental barrier. We propose two theories on how the virus reaches the fetal tissue. One, the virus infects trophoblasts, induces death and passes through the weakened barrier. Two, Rubella virus establishes a productive infection in trophoblasts and is basally secreted to underlying fetal tissue. We find that both cytotrophoblasts and syncytiotrophoblasts can be infected with Rubella virus and that a significant amount of cell loss occurs. As well, both cytotrophoblasts and syncytiotrophoblasts are able to produce infectious virus. However, virus was not released in the basal (fetal) direction unless there was a loss of membrane integrity. In conclusion, cell death and barrier destruction play the greater role in the vertical transmission of Rubella virus.

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LIST OF ABBREVIATIONS

AEC	Aminoethyl Carbazole
AIF	apoptosis-inducing factor
ATP	adenine triphosphate
Bio-UTP	Biotinylated uradine triphosphate
CMV	cytomegalovirus
CPE	cytopathic effect
CRS	Congenital Rubella Syndrome
CT	Cytotrophoblasts
DAPI	4,6-diamidino-2-phenylindole
ddH_2O	Double distilled water
DMSO	dimethyl sulphoxide
EGF	Epidermal Growth Factor
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein Isothiocyanate
H_2O_2	hydrogen peroxide
hCG	human chorionic gonadotropin
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IFN	Interferon
IgG1	Immunoglobulin 1
IH	immunohistochemistry

IMDM	Iscove's Modified Dulbecco's medium
IUGR	intrauterine growth restriction
IVIg	Intravenous Immunoglobulin
kDa	Kilodaltons
MEM	Minimum Essential Medium
MOI	multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide
ORF	open reading frame
PBS	phosphate buffered saline
PE	preeclampsia
PFU	plaque forming units
pH	negative log of hydrogen ion concentration
pi	post infection
PL	placenta
PS	phosphatidyl serine
RC	replication complexes
RT	room temperature
RV	Rubella virus
SP	signal peptide
ST	Syncytiotrophoblasts
TBM	trophoblast basement membrane
TdT	Terminal deoxynucleotidyl transferase
TER	Transepithelial Resistance

TNFα	Tumor necrosis factor α
TUNEL	TdT-mediated dUTP-biotin nick end labelling
UTP	uradine triphosphate
Z-VAD-FMK	Benzyloxycarbonyl-Val-Ala-Asp (Ome) fluoromethylketone

CHAPTER 1: INTRODUCTION & HYPOTHESIS

1.1 The Placenta

During pregnancy, the fetus grows inside the womb surrounded by a system of membranes, and the fusion of these membranes to the uterine wall forms the placenta (Figure 1). The fetus relies on the mother to provide a variety of functions that the fetus is not yet able to perform. The functions carried out by the placenta include gas transfer, excretory functions, water balance, catabolic and resorptive functions, heat transfer, immunological functions and numerous secretory and metabolic functions (12). Most of the functions occur through diffusion across the fetal cell layers in the placenta. The placenta temporarily replaces the lungs, kidney, gut, skin, endocrine glands, and liver while the fetus is developing.

1.2 Structure of the Villous Placenta

The placenta is the maternofetal contact point where both the maternal and fetal circulatory systems exist separated by only a few fetal cell layers. In humans, the fetal cell layers consist of a syncytiotrophoblast (ST) layer with underlying cytotrophoblasts (CT), and beneath this the basement membrane, placental fibroblast, villous macrophages, and lastly, the fetal endothelium of the fetal villous capillaries (Figure 2) (138). The villous trophoblasts-- ST and CT-- are specialized fetal cells that form the placental barrier. The trophoblasts invade the maternal vessels in the uterus to come in direct contact with the maternal blood. During placental development, placental villous trees, the tree-like branching of the chorion, are formed (12). The placental villous trees are bathed in maternal blood, and their outermost covering is the continuous ST. These

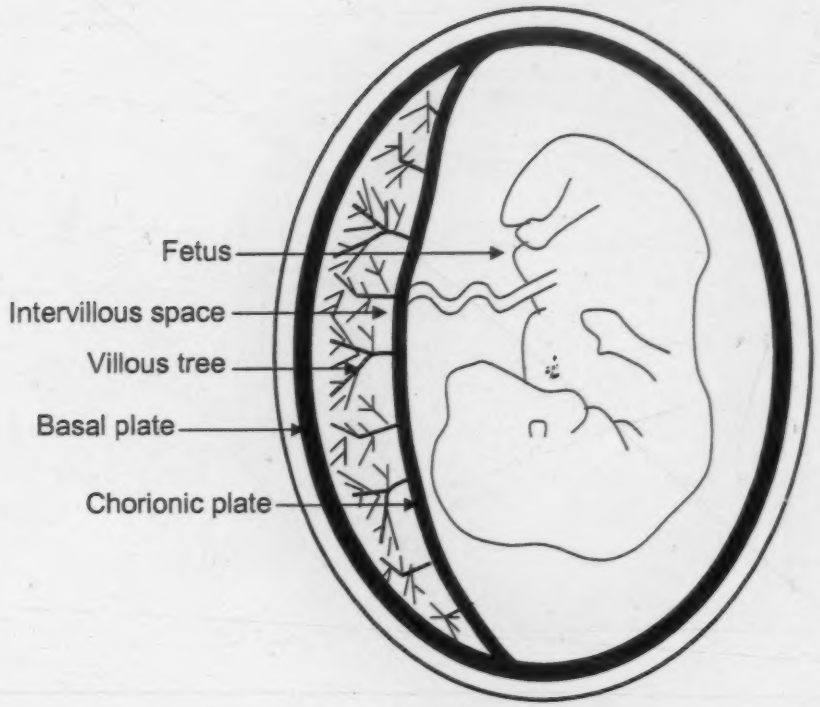


Figure 1. The structure and orientation of the human placenta. The human placenta is made up of a system of membranes that have fused to the uterine wall. The intervillous space is filled with maternal blood, bathing the villous trees. The villous trees are lined with the villous trophoblast, which protect the fetal capillaries inside. The villous trees are the exchange site for nutrients and waste, from the maternal blood to the fetal blood. (adapted from Benirschke and Kaufmann, 1990)

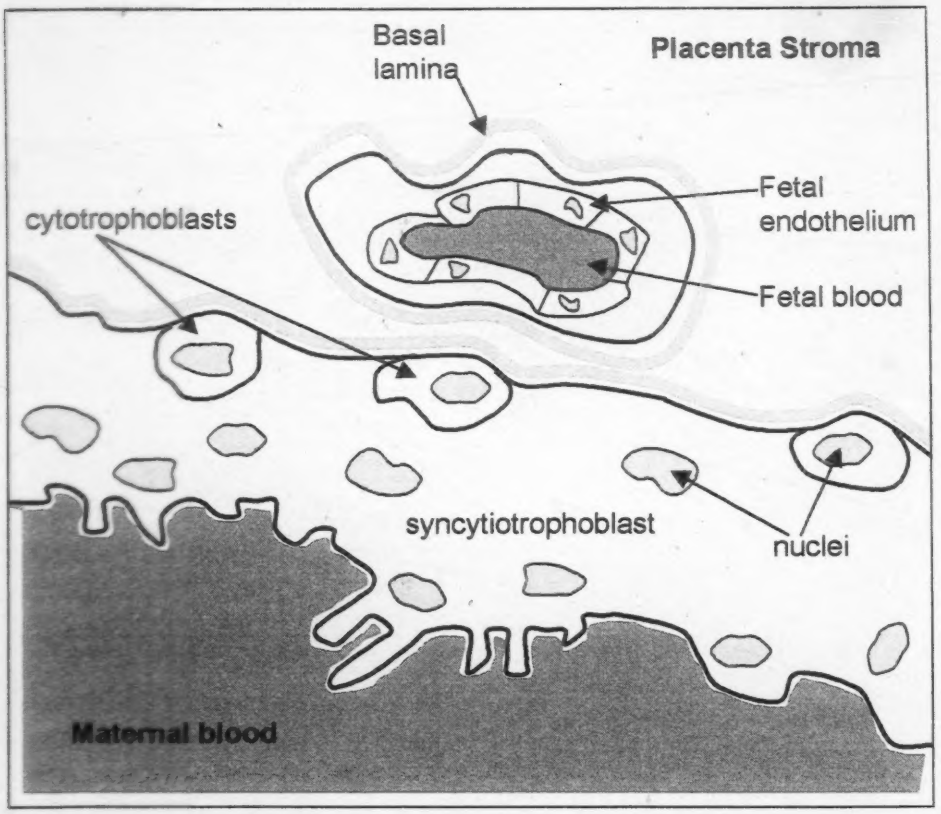


Figure 2. The placental barrier. Cross section of the villi indicating the relationship of maternal blood to fetal blood. A continuous layer of syncytiotrophoblast (ST) separates the maternal blood from the remaining fetal tissue. Cytotrophoblasts (CT) lie on the basal surface of the syncytiotrophoblast and fuse into the ST layer. The fetal endothelium surrounds the fetal capillaries that lie within the villi.

placental villous trees and the interdigitation of the tissue surfaces in the human placenta help increase the surface area, giving about 10 square meters of exchange surface at term (17).

1.2.1 The Syncytiotrophoblast (ST)

The ST is a single multinucleated, polarized cell that forms the only continuous barrier separating the maternal blood from the fetal cell layers (88). The apical surface of the ST faces the maternal blood while the basal surface lies next to the underlying CT. The surface of the ST has syncytial projections that are covered by microvillli (41). The microvillus forms the interface between the maternal blood and fetal tissues and is the exchange site for nutrients, wastes, oxygen and hormones. Being only 1-2 um thick in places, this ST layer is very efficient at transfer (17).

There are an abundant number of organelles in the electron-rich dense cytoplasm of the ST, including a well-developed Golgi, pinocytotic vacuoles, and a generous amount of rough endoplasmic reticulum, free ribosomes, and mitochondria (12). The many nuclei in the syncytium are irregularly dispersed but are often seen aggregated together in 'syncytial knots' (12). The ST functions as an endothelium, and has the ability to produce nitric oxide (45). The ST synthesizes and secretes significant levels of hormones--human chorionic gonadotrophin (hCG), progesterone, human placental lactogen (hPL)-- and glycoproteins --pregnancy-specific 1 glycoprotein (8).

1.2.2 Cytotrophoblasts (CT)

CT are villous trophoblasts that lie underneath the ST. Unlike the ST, CT are mitotically active, nonpolarized cells. Being undifferentiated and mononuclear cells, CT

contain few organelles (12). Among the few organelles seen in the undifferentiated CT are well-developed Golgi, few and few mitochondria (10, 12). As the CT differentiate, the number of organelles increases, particularly Golgi bodies, rough endoplasmic reticulum, mitochondria, and free ribosomes (12, 76). One important function of CT is to continuously replenish the syncytium, accomplished by differentiating and fusing into the above ST layer (8, 12, 113). Desmosomes are present, attaching the CT to the above ST (10, 41). The basal plasma membrane of the CT changes from being smooth with few caveolae to being irregular with villous projections (41). CT also contain receptors for epidermal growth factor (EGF) (12). Since CT are important for replenishing the syncytium, their relative number decreases as pregnancy progresses (12).

1.2.3 Culture Models

The study of the placenta involves using different culture systems: cell lines such as BeWo and JAr, primary trophoblasts from human placentas, and whole placental tissue samples (8). This range is seen because cell lines are easier to work with, yet trophoblasts and placental tissue samples provide more in vivo-like models. Using animal models to study the placenta is difficult because the placental structure varies from animal to animal and information may not be useful.

In this thesis, we studied primary villous trophoblast cells retrieved from human term placentas. The CT are obtained through trypsinization and purified through column-elimination of contaminating cells (164). The cells involved in this study are either CT-like, being primary CT cells, or ST-like. The differentiation of primary CT into ST-like cells is stimulated, in vitro, by adding epidermal growth factor (EGF) over a period of five or six days (113). Through the addition of EGF to the CT, experiments are

performed on differentiated CT that simulate the ST layer. Cell layers with exposed apical and basal surfaces are prepared by culturing ST on membrane inserts in transwell cultures (63). Doing so establishes a polarized ST-like layer that allows for the differentiation of apical and basal supernatants.

1.3 Placental Disease and Damage

Placental damage can result from maternal disorders or a specific disease. There are several different types of placental damage: placental villitis, undergrown placenta in intrauterine growth restriction (IUGR), preeclampsia (PE) infarcts, and infection (reviewed in (26, 78, 123, 126, 132, 161)). Each can result in a low birth weight for the fetus, fetal abnormalities and even fetal death. Placental villitis, sometimes referred to as inflammation of the placenta, is associated with a specific disease such as IUGR, PE, infection, or may be from an unknown etiology (7, 130). Several different factors can lead to IUGR-- maternal weight and nutritional status, infections, genetic factors, maternal stress (chemical addictions), placental anomalies, and various maternal disorders (anemia, severe chronic asthma, chronic renal disease, heart disease and hypertension) (126). With so many factors involved in IUGR, and no single cause, a diagnosis of placental insufficiency before birth can be difficult. PE is a leading cause in maternal and perinatal mortality, yet its etiology and pathogenesis are not well understood (26, 161). Hypertension and proteinuria are two tell-tale signs of PE but are not definitive (127). Infection of the placenta accounts for 3% of congenital anomalies and can result from several viruses, including Rubella virus (RV), Cytomegalovirus (CMV), Herpes Simplex Virus (HSV), and Varicella-zoster virus (35). Most infections lead to placental villitis. The relationship between infection and IUGR or preeclampsia is

full of unknowns, and several reviews suggest a link (9, 153, 155). However, one study indicated no relationship between IUGR and RV infection (82).

1.3.1 Apoptosis in the Placenta

Increased apoptosis is one mechanism of damage seen in the placenta. In a normal placenta, continuous apoptosis, which regulates the number of cells, occurs (74, 144). Several researchers have documented apoptosis in the placenta, specifically in the ST and the villous stroma (50, 144). During regular remodelling of the syncytium layer, apoptosis occurs. The trophoblasts are continuously changing, from proliferating CT to maturing CT, from maturing CT to ST, and from ST to dying trophoblasts. This process occurs over several weeks. While apoptosis is a normal process continuously occurring in the placenta, in diseases such as IUGR and PE, the amount of apoptosis increases (97, 143). The exact mechanisms and causes of apoptosis vary and are not always known. TNFα is one factor that is thought to play a role in placental apoptosis (90, 165). ST are known to undergo increased apoptosis due to TNFα stimulation. EGF is one growth factor that has been shown to inhibit apoptosis in trophoblasts when apoptosis was induced by ceramide or cytokines such as TNFα and IFNγ (53, 124). During various infections in the placenta the amount of apoptosis is undetermined; however, apoptosis is known to be a consequence of viral infection in some cells (50, 73, 148).

1.4 Vertical Transmission

Vertical transmission is the passage of infectious agents from the mother to the growing fetus. There are two main mechanisms of transfer through the placenta:

ascending infection and intrauterine transplacental infection (138). Ascending infection occurs when microbes in the cervix reach the placenta. Intrauterine transplacental infection takes place when infectious agents in the maternal system reach the fetus through the villous placenta or the extravillous placenta. Even though the transplacental passage of viruses is a critical aspect of congenital viral infection, the placental viral infection and the role placental villous trophoblast cells have in the passage are not well characterized. This study looks at the potential roles of the villous placenta and villous trophoblasts in the transplacental passage of RV. Koi et al. (2001), while looking at adenovirus, HSV, and human immunodeficiency virus (HIV) suggested that the villous trophoblast is relatively resistant to viral infections because of decreased expression of viral receptors (87, 88). However, different viruses have different cell surface receptors. As well, very little is known about how the placental viral infection leads to the following pathologic consequences: fetal anomalies, intrauterine fetal death, and persistent postnatal infection.

It may also be of interest to note that viruses are not the only teratogens. Some chemicals and drugs such as vitamin A and D, aspirin, and thalidomide are capable of causing severe damage to the fetus through transplacental delivery (15).

1.4.1 Specific Infectious Agents

Women must be aware of the variety of agents that have the potential to cross the placenta and damage the fetus during pregnancy. Several viruses infect the fetus through the placental route after maternal infection, including the Hepatitis B virus, Hepatitis C virus, RV, CMV, Varicella-zoster virus, and HSV (35, 88). Other infectious agents that infect through the placenta include some parasites (*Toxoplasma gondii* and *Trypanosoma*

cruzi), fungi (*Candida albicans*) and bacteria (*Listeria monocytogenes*) (45, 138). Viral infection causes fetal anomalies due to the following primary mechanisms: cell death, abnormalities of cellular growth, chromosomal damage, and secondary inflammatory responses (35). These fetal anomalies include pregnancy loss because of stillbirth, spontaneous abortion or premature delivery, physical anomalies, and postnatal persistence of infection (35).

1.5 Rubella Virus (RV)

Rubella was first described as a unique disease in the late 1800s and the virus was isolated in cell culture in 1962 (48). RV is the only member of the Rubivirus genus and belongs to the *Togaviridae* family. RV causes Rubella or German measles, a relatively mild disease in children and adults. The characteristic symptoms of this disease are a rash and a fever; however, nearly half of the infections are assymptomatic (35, 92, 159). RV is a major teratogen, causing severe congenital abnormalities to a fetus, especially when maternal infection is present during the first trimester of pregnancy (48, 158).

1.5.1 Physical and Genomic Structure of RV

RV is a relatively small, spherically shaped, positive single-stranded RNA virus about 60-70 nm in diameter (23, 85). The nucleocapsid is surrounded by a lipid envelope that is acquired during budding through intracellular membranes or through the plasma membrane (64, 69). RV has a 40S genomic RNA and produces a 24S subgenomic RNA during replication (102). The 40S RNA encodes for the nonstructural proteins and serves as a template for the production of the 24S RNA.

1.5.2 Replication of RV

The RV attaches to the cell membrane through an undetermined cell surface receptor. However studies indicate that there may be more than one receptor, and potential receptors include phospholipids –phosphatidylinositol, phosphatidylcholine, and sphingomyelin –and a glycolipid –cerebroside sulphate (110, 111). Once attached the virus can then enter the cell through receptor-mediated endocytosis (48) (Figure 3). Once inside a low pH endosome, membrane fusion occurs, allowing the capsid and genomic RNA access to the cell cytoplasm (48, 80, 125). RV replicates in the cytoplasm of infected cells, with the aid of host cell proteins. During replication, vacuoles form inside the infected cells, termed replication complexes (RC) (94, 95). The RC are in the cytoplasm of the cell and are associated with the rough endoplasmic reticulum (95). The inner membrane of the RC is lined with spherical structures (107), which is the site of viral RNA synthesis (92).

Since RV has a positive strand of RNA for its genomic poly nucleic acid, there is no need for synthesis of an mRNA, and protein synthesis can begin immediately. Production of the polyprotein P200 begins by translation of the 40S RNA at the 5' end open-reading frame (ORF). This P200 is cleaved via an internal, papain-like cysteine protease to produce the two non-structural proteins, P90 and P150 (22, 44, 100, 108). P150 is located on the N-terminus of P200, P90 on the C-terminus, and the protease is located on the C-terminus of P150. Replication continues with the production of a full length minus strand from which the 24S subgenomic RNA is made, as well as the full-length viral 40S RNA that is destined for encapsidation (Figure 4). Viral RNA synthesis is first detected at 12 hours post infection (64, 140). The ORF on the 24S RNA is then

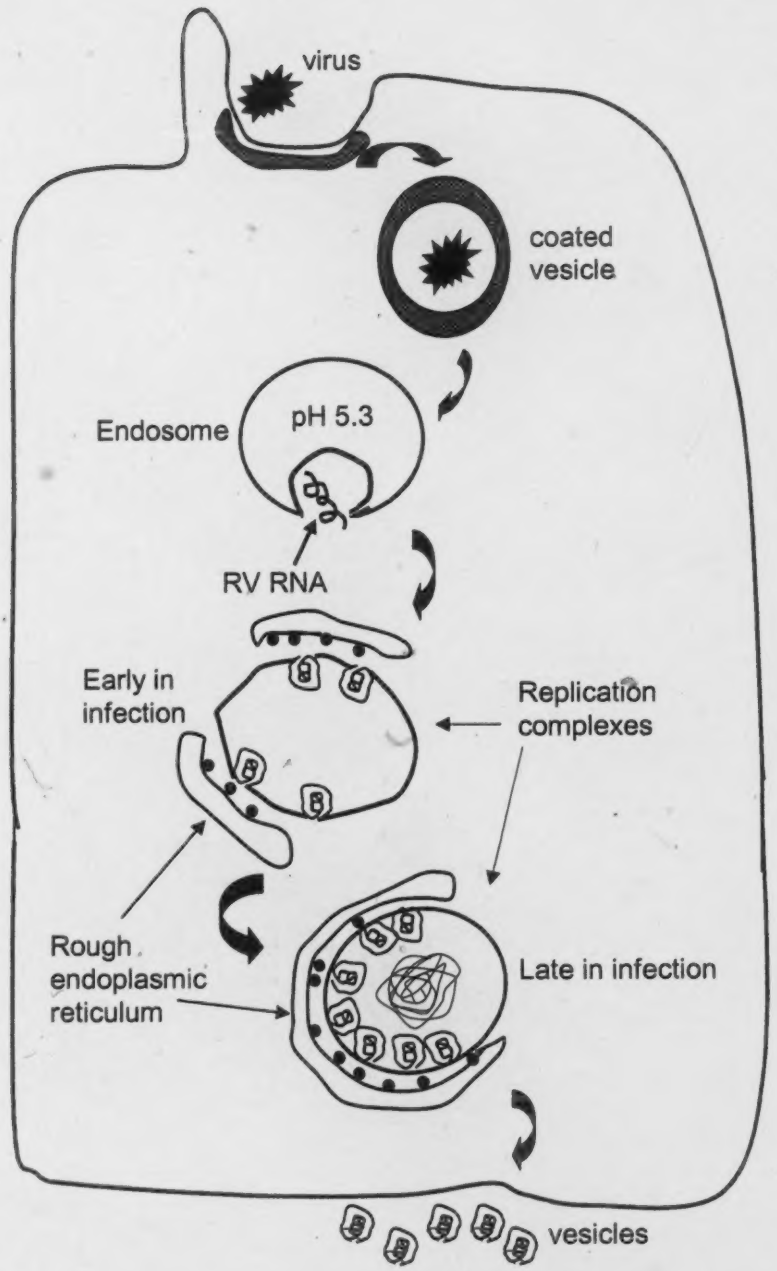


Figure 3. Rubella Virus Replication Cycle. Rubella virus attaches to the cell surface, is endocytosed into a low pH vesicle, the RNA then obtains access to the cytoplasm, and begins replication. The five viral proteins are produced, viral assembly begins, and progeny virus is released into the cytoplasm. (adapted from Frey and Wolinsky, 1999)

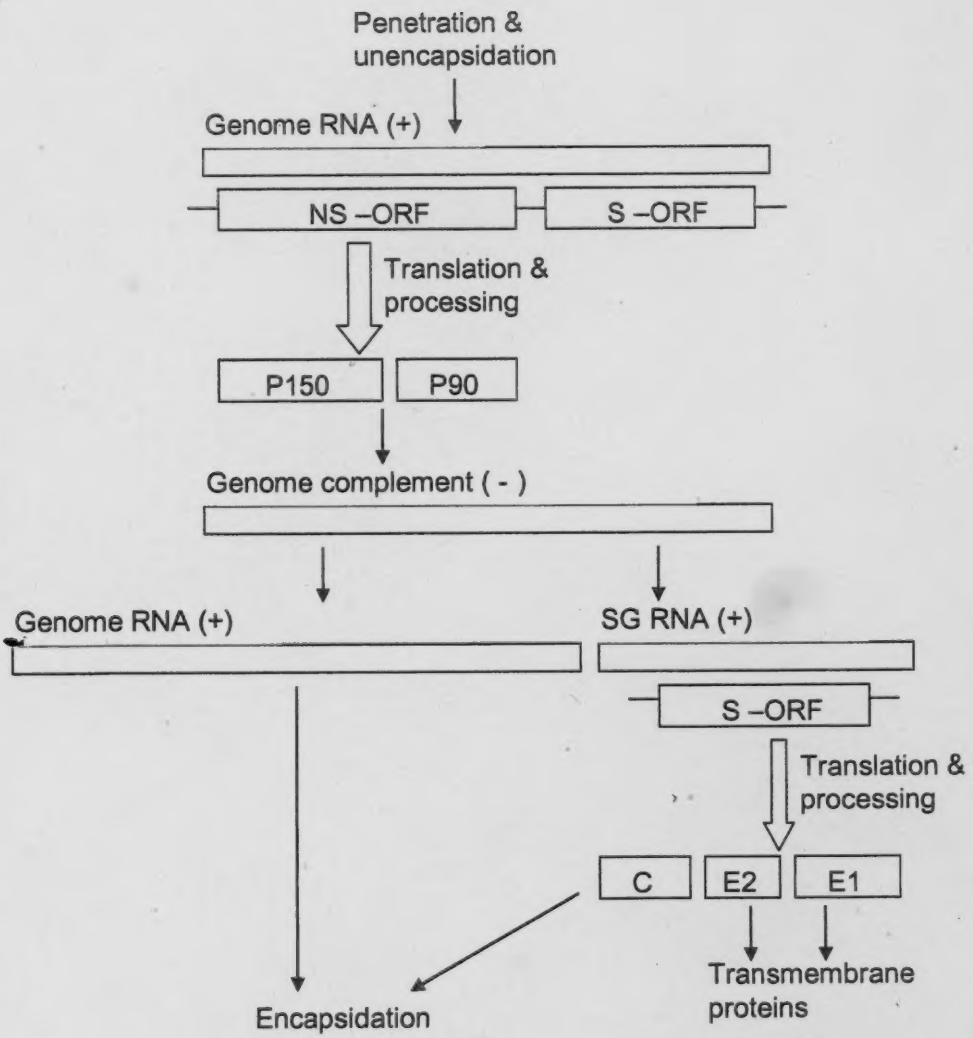


Figure 4. Rubella Virus RNA Replication and Protein Production. The nonstructural open reading frame (NS –ORF) is processed first to produce two nonstructural proteins, P150 and P90. Then the genome complement is produced and from this a replicate strand of RNA and a subgenomic RNA (SG RNA) strand are created. On the SG RNA strand is the structural open reading frame (S –ORF) from which the three structural proteins, C, E2, and E1 are produced. (adapted from Frey and Wolinsky, 1999)

translated to generate another polyprotein that is cleaved to produce three structural proteins: Capsid (C), E2, and E1 (119). All three structural proteins are translated together as a polyprotein precursor and cleaved by a cell protease, signal endopeptidase (118, 119).

C, E2, and E1 are translated on the endoplasmic reticulum (ER) (70). There are two signal peptides (SP) in the polyprotein precursor, E2 SP and E1 SP (66, 67). E2 SP directs translocation of the two glycoproteins into the lumen of the ER. From the ER, the proteins are then transported to the Golgi (68, 71, 72). As the virus matures, it buds from either the plasma membrane or internal cellular membranes, depending on the cell type, and the lipid membrane is formed (69, 95). The newly formed virus is then ready for release from the cell. RV can replicate and be released within 24 hours (64).

1.5.3 RV-encoded Proteins

There are two non-structural proteins: P150 (150 kDa) and P90 (90 kDa). The functions of these two non-structural proteins, P90 and P150, have not yet been fully elucidated. The non-structural proteins are necessary for structural protein synthesis and viral replication (99, 128). The predicted functions of these proteins include P150 with methyltransferase activity (MT) and P90 with a helicase (H) domain and a RNA polymerase domain (R) (48, 92).

There are three structural proteins, C, E2 and E1. The C protein (~34 kDa) or capsid protein forms the viral nucleocapsid by making several disulfide-linked homodimers (48, 93). This protein is responsible for packaging genomic RNA inside the nucleocapsid (102). The E2 and E1 proteins are glycoproteins that are embedded in the viral envelope

(69, 95, 118). E1 (~59 kDa) contains the viral hemagglutinin site (66, 157). The cytoplasmic domain of E1 is needed for virus release from the cell (163).

1.5.4 RV and Apoptosis

Apoptosis or programmed cell death is a mechanism that is triggered by a variety of stimuli and ultimately leads to death of the cell (139, 148). Apoptosis is necessary for normal development, host defense, and the suppression of oncogenesis (73, 117, 146, 154). Apoptosis, regardless of the pathway ultimately leads to death of the cell with these characteristic signs: degradation of nuclear DNA, cytoplasmic shrinkage, movement of phosphatidyl serine (PS) to the outer membrane, and membrane blebbing (146, 154, 166). Methods for the detection of apoptosis include TdT-mediated dUTP-biotin nick end labelling (TUNEL; which labels the nicked and damaged DNA), DNA laddering with gel electrophoresis, PS staining with annexin V (146, 154). It is important to note that all the machinery needed for apoptosis is present in the cell in an inactive state waiting for stimulation (146, 166). Apoptosis is initiated by many different stimuli, and leads to activation of receptor molecules (often members of the TNF receptor superfamily), recruitment of adaptor proteins (such as TRADD or FADD), and recruitment and activation of caspases (27, 32, 146, 166). Apoptotic stimuli include unscheduled DNA synthesis, UV irradiation, viral infection, drugs and toxins, growth factor withdrawal, or ligand binding to specific surface receptors (often members of the TNF receptor superfamily) (117, 137, 146, 148, 154). Caspases are a family of cysteine proteases, with at least 14 different caspases identified in mammals (27, 116, 146). The specific caspases involved in apoptosis depends on the stimulus and cell type involved. Caspases are synthesized as inactive pro-forms or zymogens and are activated through

cleavage (146, 166). Cleavage of the zymogen results in a heterotetramer with 2 identical subunits of ~20 kDa and 2 identical subunits of ~10 kDa (134, 146, 156, 160). Caspases, once cleaved, are active enzymes that can cleave substrate proteins at an aspartate residue, and are capable of activating themselves (27, 146). Once initiator caspases, such as caspase 8 or caspase 10, have been activated this leads to further activation of caspases, ending with the activation of effector caspases, such as caspase 3 or caspase 6 (146, 166). Apoptosis usually leads to caspase activation that converges on the activation of nucleases, which are responsible for the degradation of nuclear DNA, such as cleavage of iCAD from CAD leaving CAD active to cleave internucleosomal DNA (40, 101, 136, 146). Active caspases are also responsible for inactivating cellular proteins such as DNA repair enzymes, and directly or indirectly activating enzymes (116, 146, 149). Ultimately the apoptotic cell is phagocytosed. The mitochondria also have an important role in apoptosis. During apoptosis the membrane permeability increase resulting in release of cytochrome c and other pro-apoptotic molecules (27). Cytochrome c activates the caspase cascade by forming a complex with Apaf-1, ATP, and pro-caspase-9 (31, 46, 84, 98, 166). This multi-protein complex is called an apoptosome and activates caspase 9. The execution of apoptosis can also involve increased activation of p53, JNK activation, NFκB activation depending on the stimulus and cell type (56, 146). NFκB and JNK are transcriptional regulators and can increase the expression of both proapoptotic and antiapoptotic proteins (146). NFκB can increase the expression of Fas, p53, Bax and others that will activate caspases and promote apoptosis (56). There are also cellular proteins and factors that promote or inhibit the apoptotic pathway and their ratio is important in whether or not the cell dies. These inhibitors include members of the Bcl-2

family such as Bcl-x_L (13) and Bcl-w (55), and growth factors (139, 146, 154). These anti-apoptotic Bcl-2 proteins are present on the mitochondrial membrane (27, 33). Members of the Bcl-2 family are also responsible for promoting apoptosis, such as Bcl-x_S (13), Bax (120), and Bak (25, 42, 146). These pro-apoptotic proteins are present in the cell cytosol. The Bcl-2 family of proteins regulates the release of cytochorme c from the mitochondria as well as the aggregation of adaptor proteins and indirect caspase activation (27, 166). There is one method of apoptosis that doesn't involve the caspase cascade, but still involves the mitochondria –the release of apoptosis-inducing factor, AIF (18, 19, 84, 103). Upon receiving a self-destruct signal, neurons and other cells allow AIF to be released from the mitochondria, after which it migrates to the nucleus, binds to DNA and triggers destruction of DNA and cell death (84, 103). The combination of inactive and active proteins, the ratio of apoptotic inhibitors and promoters, and the genes actively being transcribed within the cell, determine whether that cell dies through apoptosis.

Viruses are capable of inducing and inhibiting apoptosis. There are several RNA and DNA viruses such as Sindbis virus, HIV-1, Parvovirus, and SV40 in which apoptosis is seen as a consequence of their replication (148). Some viral proteins known to suppress apoptosis are CrmA from cowpox virus, IE1 and IE2 from CMV, SPI-2 from vaccinia virus, and Large T from SV40 (148, 154). In many primary cell cultures and continuous cell lines, RV produces little cytopathic effect (CPE) (47, 64, 85, 129). However, in Vero cells (African green monkey kidney cells), RK-13 cells, and BHK-21 cells, RV induces CPE and apoptosis (5, 38, 39, 73, 112). RV-induced apoptosis is shown to be caspase-

dependent in both Vero cells and oligodendrocytes in rat glial cell cultures in vitro (36, 129).

1.5.5 Tropism

Several cell lines have been used to study RV: Vero (African Green Monkey Kidney), RK13 (Rabbit Kidney), and BHK21 (Baby Hamster Kidney) (23, 85). One study reported that RV could persistently infect several different cells: Vero, RK13, BHK21, BSC-1, HeLa, and rabbit embryo chondrocyte (145). It has also been reported that RV can infect human lymphocytes isolated from peripheral blood, specifically the T cell population, after stimulation with a mitogen such as phytohaemagglutinin (21).

Two compounds have been shown to have an effect on RV production—interferons (IFN) and actinomycin D. The presence of IFNα and IFNγ was seen to have caused a decrease in the amount of viral protein synthesis (114, 162). Vero cells lack an IFN response, due to loss of the IFN α and β genes (34), and therefore RNA viruses, such as RV, can grow uninhibited (24). However, trophoblasts, especially syncytiotrophoblast, produce interferons and may produce other molecules that have an antiviral effect (2, 3, 17). Actinomycin D has a small time-dependent effect on RV replication. Actinomycin D or alpha-amanitin, two transcription inhibitors, may inhibit essential cellular factors that are used in viral replication (114).

1.6 Congenital Rubella Syndrome (CRS)

Rubella was considered only a nuisance until the 1940's when Norman Gregg, an Australian ophthalmologist, associated RV infection in pregnancy with developmental abnormalities present in some infants (158). Further investigation in the 1940's

confirmed that contracting Rubella during the first trimester of pregnancy poses a risk to the fetus of developing congenital abnormalities (reviewed in (158)). The last major epidemic in North America, in 1964, left the United States with 20 000 CRS cases and led to the rapid development of a vaccine (48, 79, 92, 135). The vaccine for the RV was first used in vaccination programs in Canada, the United States, Japan, and several European countries in 1969 (48).

Research done in the 1950's and 60's using pathology showed that the RV does infect the placenta, trophoblasts, and embryonic tissue (37, 57, 141, 150). Most of the early studies involved looking at the range of damage seen in aborted fetuses and infected placental tissue (141, 150). Tondury and Smith stated, "abnormal lesions were noted microscopically in 8 of the specimens and consisted of scattered foci of damage in the syncytiotrophoblast and cytotrophoblast cells with pyknotic nuclei and swollen cytoplasm in the affected cells" (150). As well, Golda Selzer concluded that the virus invaded embryonic tissue as evidenced by the cell degeneration seen and that this was "a fundamental event in the pathogenesis of fetal abnormalities due to maternal rubella" (141).

1.6.1 Characteristics of CRS

RV causes a characteristic set of developmental abnormalities in the fetus called Congenital Rubella Syndrome (CRS). These developmental abnormalities include blindness and cataracts, neurosensory deafness, congenital heart problems, and, when severe, neurological abnormalities (23, 158). There may also be some transient abnormalities such as low birth weight, hepatospenomegaly, and thrombocytopenic

purpura (23). The risk of congenital effects if the mother has a RV infection during the first 11 weeks is 90% (58).

RV and other viruses cause the various abnormalities in the fetus as a result of four primary mechanisms: cell death, abnormalities of cellular growth, and secondary inflammatory responses (35). Whether or not the fetal abnormalities seen are a result of damage due to RV-induced apoptosis is not clear, but it is a possibility.

1.6.2 Rubella Epidemiology

Since the introduction of vaccination programs in 1969, the incidence of Rubella has significantly decreased, but has not been eliminated (131, 147). Several studies indicate that a portion of the population is still susceptible to RV infection; the exact amount varies worldwide from 4% to 70% depending on the region (6, 29, 30, 48, 58). It is estimated that 10-20% of young adults and women of childbearing age are susceptible to Rubella in regions with vaccination programs (23, 35, 115). As well, immunization is not necessarily permanent, and reinfection is possible (4, 16, 28, 81). During an outbreak in Manitoba in 1996, 31% of the females who contracted Rubella had been previously immunized (59). Immigration also plays a role in the continuation of the RV, especially since most developing countries do not have vaccination programs in place. A Bulletin from the World Health Organization in 1997 indicated that only one third of all countries have a Rubella vaccination policy and that most of the countries in South America, Africa, and Asia as well as a few other countries lacked a Rubella vaccination program (133). Rubella has continued to persist for the past 15 years despite a good vaccination program in Canada for the past 30 years (147). Rubella may be around for a long time to

come, given that there are still susceptible people including highly susceptible immigrants, and the possibility of secondary infection (131).

1.7 Vertical Transmission of RV

Although we know that RV can infect the placenta resulting in significant damage to the growing fetus, there are many things we do not know (48, 52). For instance, we do not know how RV and the trophoblast interact and how the virus reaches the fetus. There are no details on RV infection of the ST, specifically whether the infection is productive or induces death.

We do know that RV is able to infect polarized cell lines, both from the apical and basal surfaces (51). However, the direction of productive virus release varies within different cell lines. RV is able to infect and be secreted from both the apical and basal surfaces of some polarized cell lines including Vero C1008 (51). The direction of infection and viral release of the RV in the trophoblast is not known.

1.8 Rational

The studies by Tondury and Smith (1966) and Selzer (1963) as well as others (37, 57, 121) indicate that the placenta and fetal tissue are infected, supporting further investigation into the details of the RV/trophoblast interaction.

As well, there are two main reasons why the study of the RV is important and should continue. First, the study of the interaction between the RV and the trophoblast will provide us with information that will be useful when looking at other infectious agents. The study of how infectious agents reach the fetus is useful, and RV can prove to be a good model for other infections.

Second, RV continues to infect people, even though a vaccine against it was put into use in 1969, with several outbreaks occurring in North America in the past ten years (59, 60, 151, 152). As a result, pregnant women are at a great risk. Since the 1970's, the number of CRS cases has declined but has not been reduced to zero even in countries that have comprehensive vaccination programs (60, 115, 131, 147, 151, 152).

1.9 Hypothesis

Although we know that RV is able to induce severe damage in the fetus, how the virus reaches the fetus and induces this damage is unknown. One way of entry may be through the villous placenta, where the fetal blood can be only 2 μm from the maternal blood at times, and the maternal blood is continuously in direct contact with fetal cells. There are two main mechanisms by which the RV may reach the placenta, either through basal release as a consequence of RV infection of the polarized ST, or through barrier damage resulting from significant cell loss during RV infection of the ST and CT. However in primary cell cultures, RV replicates slowly and to relatively low titers and consequently produces little or no CPE (47, 129). Therefore, it remains to be seen, in primary trophoblasts, to what extent RV can replicate and the degree of CPE induced.

1.10 Experimental Approaches

1.10.1 Determine whether trophoblasts are infected productively

First it is important to establish whether CT and ST can be infected. Then whether or not the trophoblasts can produce active virus needs to be determined. If progeny virus is produced and released, then the direction of this release, whether apical or basal, must be determined. Confluent membrane culture inserts will be used to aid in determination of

basal release. The direction of release cannot be assessed until the amount of cell death is determined to ensure leakage is not mistaken for directional release.

1.10.2 Determine the amount of cell death

Once it is confirmed that trophoblasts may be infected and infection methods are established, the percentage of death wills to be determined. If there is a large amount of cell death, this needs to be documented and both the mechanism of cellular death and the amount of barrier destruction will be investigated. If however, there is a low amount of cell death, then the integrity of the barrier and the direction of virus release will be determined. In determining the method of cellular death, the effect of apoptotic inhibitors, such as Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) (129, 142) and anti-TNFα (20) will be investigated. Ultimately, it may be possible to limit virus-induced cell death with growth factors such as EGF (53, 124).

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Culturing of Cell Lines

Vero 76 (African Green Monkey Kidney) cells and RK13 (Rabbit Kidney) cells were provided by Dr. Tom Hobman (Department of Cell Biology, University of Alberta) (89). Vero cells were cultured in Iscove's Modified Dulbecco's medium (IMDM; Gibco BRL, Grand Island, NY), supplemented with 5% fetal bovine serum (FBS; Gibco BRL, Rockville, WI) heat-inactivated for 30 minutes at 60°C, 100 U/ml penicillin (Gibco BRL, Grand Island NY), 100 μg/ml streptomycin (Gibco BRL, Grand Island NY) and 292 μg/ml glutamine (Gibco BRL, Rockville, WI). The cells were cultured in a humidified incubator (Napco CO_2 Incubator 5420-0) at 37°C and 5% CO_2. RK13 cells were grown in Minimum Essential Medium (MEM; Gibco BRL, Grand Island, NY), supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml glutamine, and 1x MEM non-essential amino acid solution (Sigma Chemical Co., St. Louis, MO) at 37°C in 5% CO_2.

2.1.2 Isolation of Primary Villous Placental Trophoblasts

Trophoblasts were isolated from human term placentas obtained from patients following normal delivery or elective caesarean section. Trophoblasts were isolated using column purification to >99.99% purity as previously described (83, 164). The chorionic tissue was minced and subjected to 7 trypsin (0.25%)/Dnase (5 units/ml) digestions of 8 minutes each. Solution containing cells in suspension were removed and

fresh trypsin/Dnase added between each digestion. Cells from fractions 3 to 7 were pooled and washed 2x in PBS containing 2% fetal calf serum (FCS). Cells were resuspended in lysis buffer containing 150mM ammonium chloride, 10 mM sodium bicarbonate, and 0.1 mM disodium EDTA and subjected to intermittent inversion for 8 minutes at RT. Following this, cells were washed 2x with PBS containing 2%FCS and incubated in PBS containing 20% normal goat serum on ice for 20 minutes. Cells were then washed and resuspended in PBS/2%FCS. Since trophoblasts do not express CD9, MHC class I, or MHC class II, negative selection was used to remove contaminating cells expressing these markers. The cell mix was incubated with antibodies to CD9 (Clone 50H.19; (106)), MHC class I (W6/32; Harlan Sera-Lab, Crawley Down, Sussex, England) and MHC class II (Clone 7H3; (106)) for 40 mintues on ice, after which cells were washed 2x with PBS/2%FCS and resuspended at 1 x 10^7 cells/ml in PBS/2%FCS. Plastic columns (Perkin Elmer Wallac Inc.) were loaded with a 1/32-inch porous polyethylene filter grade frit (Atlas Mineral Chemical Co., Mertztown, PA) and 1.5 ml of prepared glass beads. The glass beads (Sil Industrial Minerals, Edmonton, AB) were passed through a series of sieves (Fisher Scientific, Fair Lawn, NJ) to isolate beads of a uniform size. The glass beads that passed through a 60 mesh sieve and an 80 mesh sieve were retained and from this the beads that passed through a 100 mesh sieve were discarded. The beads were acid washed with 100 ml concentrated HCl and 100 ml concentrated HNO_3 per 200 ml glass beads for at least 15 minutes then washed 6x with tap water and 4x with ddH_2O. Next, the beads were washed 1x with 0.15 M NaH_2PO_4 buffer, and then 100 ml fresh buffer added and the beads were autoclaved. The beads were incubated with mouse Ig (Sigma Chemical Co., St. Louis, MO) in PBS for 48 hours

at 4°C, washed with PBS, and stored in PBS at 4°C. After the beads were loaded in the columns they were incubated with 10 mg/2 ml/column goat anti-mouse Ig-conjugated antibody (E-Y Laboratories) for 45 minutes. The columns were washed with PBS and flow rate adjusted to 1 drop/6 seconds. Cells were loaded onto columns to a maximum of 10^8 cells/column. Non-adherent cells were eluted with 13 ml of PBS/2%FCS, then centrifuged and resuspended in 100% FCS at 1x 10^7 cells/ml. The cells were cryopreserved in calf serum (GibcoBRL, Grand Island, NY) containing 10% dimethyl sulphoxide (DMSO; Caledon Laboratories Ltd., Georgetown, ON, Canada) and stored in liquid nitrogen. To confirm a high purity in each cell preparation, the cells were cultured for 24 hours as CT, fixed with methanol for 10 minutes at 4°C, and immunohistochemistry carried out with anti-vimentin (1:50; Dakopatts, Glostrup, Denmark) using an histostain kit (Zymed Laboratories Inc, San Francisco) (164). All preparations had >99.99% purity with <6 vimentin positive cells per well. Trophoblasts do not stain positive for vimentin (86), a class of intermediate filament proteins known to be present in endothelial cells and fibroblasts (122).

2.1.3 Culture of Primary Villous Trophoblasts as CT

Once thawed, the trophoblasts were cultured in IMDM (Gibco BRL, Grand Island, NY) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Trophoblasts were plated at 1.0-1.5 x 10^5 cells per well and incubated for 4 hours at 37°C in humidified 5% CO_2. Following this incubation, the cells were washed 2-3x by gently pipetting the media around the edge of each well. The CT were cultured for twenty-four hours at 37°C in humidified 5% CO_2 before treatment. The media on the CT were changed every 2 days.

2.1.4 Culture of Primary Villous Trophoblasts as ST

To stimulate differentiation of CT into ST, human EGF (Prepro-Tech, Rocky Hill, NJ)) was added at 10 ηg/ml to the growth medium as previously described (53, 113). The ST were cultured for 5 days at 37°C in humidified 5% CO_2 prior to any experimental treatment. ST were continuously cultured in IMDM containing 10 ηg/ml EGF and the media was changed every 2 days. In vitro, EGF stimulates differentiation of CT into ST patches – continuous layer of multinuclear cells.

2.2 Rubella Virus Stocks

2.2.1 Rubella Virus Propagation

RV (wild type strain M33) (89) was obtained from Dr. Tom Hobman (University of Alberta) and propagated in Vero cells as previously described (64). Briefly, 1.5×10^7 Vero cells were infected at a multiplicity of infection (MOI) of 1.0. At day 3 through to day 6 post-infection (pi) the supernatant containing virus was collected, aliquoted and stored at -70°C.

2.2.2 Determining Rubella Virus Titer

Two different methods for determining virus titer were used for this study. The first method was the plaque assay (89). For the plaque assays 3×10^5 RK13 cells/well were plated in a 6-well dish and incubated for 24 hours at 37°C in 5% CO_2. Serial dilutions of the virus were made in MEM, from 10^1 to 10^7. Prior to infection, the cells were washed with PBS, then 2 ml of the virus was added, and the cells were incubated for 4 hours at 37°C in 5% CO_2. Following infection, innoculum was removed, the cells washed with PBS, and fresh medium was added. After one hour the medium was removed and 3 ml of

0.5% agarose (Gibco BRL, Grand Island, NY) in 2x MEM media was added per well. The plates were then incubated for 2 days, turned upside down and incubated for an additional 4 days. The plates were then stained for 2 hours in MEM media containing 11.5mM Neutral Red (Gibco BRL, Grand Island, NY) and plaques were counted. The virus titers were calculated based on the number of plaques and the dilution factor of virus added to that well.

The second assay used to determine the virus titer was immunohistochemistry (IH). This assay stained the viral E1 glycoprotein envelope antigen of infected cells (65). IH for virus E1 envelope glycoprotein was carried out using a histostain kit (Zymed Laboratories Inc, San Francisco). The Vero cells were plated at 1 x 10^4 per well in a 96 well plate (Nunc, Roskilde, Denmark) and cultured for 24 hours. Serial dilutions of the virus were made in IMDM, from 10^1 to 10^7, 100 µl was added per well, and the cells incubated for 24 hours at 37°C in 5% CO_2. The cells were fixed with 100% ice-cold methanol (Fisher Scientific, Fair Lawn, NJ) for 10 minutes at 4°C. The cells were then washed with PBS and 50 µl of 3% hydrogen peroxide (H_2O_2; Caledon Laboratories Ltd., Georgetown, ON, Canada) was added per well for 10 minutes at RT. The H_2O_2 was removed and 40 µl of blocker solution (10% non-immune goat serum) supplied by the manufacturer was added for 30 minutes at RT. Following this the cells were washed 2 x with PBS and 40 µl of primary antibody: either B2, the mouse monoclonal to the RV E1 glycoprotein, at 5 µg/ml (Dr. Barbara Pustowoit, Leipzig, Germany) or an IgG1 isotype control at 5 µg/ml (Dako Corporation, Carpinteria, CA) was added per well for 30 minutes at RT. The cells were quickly washed 4x with PBS and then washed 2x with PBS for 5 minutes on a shaker. Following this, 40 µl of a biotinylated secondary

antibody (Zymed Laboratories Inc, San Francisco) was added for 30 minutes at RT. Then the cells were washed 6x with PBS as described above. Peroxidase conjugate (40 µl; Zymed Laboratories Inc, San Francisco) was added for 10 minutes at RT and the cells washed 6x with PBS. Then Aminoethyl Carbazole (AEC) substrate solution containing 1x concentration of substrate buffer, chromogen solution and H_2O_2 solution was freshly prepared from an AEC kit (Zymed Laboratories Inc, San Francisco) and 50 µl added to each well. Once the stain developed (approximately 1-4 minutes) the cells were washed 3x with PBS, PBS was added to the cells, and the cells were stored at 4°C in the dark until evaluated. The AEC substrate yields a dark red precipitate indicating positive stain. Once the staining assay was complete, the virus titer was calculated based on the number of stained foci, which I equated to RC, and the dilution factor of virus added to that well (titer of stock = # of RC/volume added in ml x dilution factor). I have equated RC with plaque forming units (PFU) (see Results section 3.1); thus, virus titer in subsequent methods is expressed in PFU.

2.3 RV Infection of CT and ST

Once CT and ST were plated and ready for treatment, 100 µl/well of RV (1.6 x 10^7 PFU/ml) was added to the existing media in each well and incubated for 24 hours at 37°C in 5% CO_2. Then the virus was removed and 100 µl of fresh media was added per well. ST were cultured in ST media throughout infection. The media was changed every 2 days post infection (pi). The trophoblasts were fixed with 100% ice-cold methanol for 10 minutes at 4°C on different days pi. In order to determine the amount of infection in trophoblasts, IH was used as described in section 2.2.2. Infection was evaluated by

counting the number of positively stained RC and quantified by dividing the RC by the total number of nuclei calculated. Trophoblasts form multinucleated cells, and for this reason, expressing infection as RC per cell would be misleading and more difficult to interpret.

To obtain an estimate of the number of nuclei per well, the nuclei were stained with 50 µl per well of hematoxylin solution (Sigma Diagnotics, St. Louis, MO). The number of nuclei in a square grid was counted in five areas of the well. The same five areas were counted in each well. A calculation was then made to approximate the total nuclei per well: (total # nuclei in all five grids/5) x # of grids in the whole well at the specific magnification power (example 132 grids/well for 20x magnification). The total nuclei per well were calculated, triplicate samples averaged, and infection quantified.

2.4 RV Production and Release from Trophoblasts

2.4.1 Testing Trophoblasts Supernatants for Rubella Virus

To determine if CT and ST were able to produce RV, both supernatants and cellular lysates were tested for progeny virus. Cultures were prepared as previously described (section 2.1.3). CT and ST were infected with 100 µl/well of RV (1.6×10^7 PFU/ml) and cultured for 24 hours. The innoculum was removed, the cells gently washed with RT PBS, 100 µl of fresh medium added to each well and the incubation continued for another 24 hours. The media was changed every 2 days thereafter. The supernatants were removed on days 2, 4, 6 pi and stored at –70°C. At each time point, after removing the supernatant, the cells were washed 3x with RT PBS, then 200µl of media was added to

each well and the plate sonicated for 5 minutes. Each well was then scraped and the media containing cellular lysate was collected and stored at –70°C. After thawing, 50 µl of each supernatant or lysate sample were added to Vero cells, IH staining carried out as in section 2.2.2, and the total number of RC counted per well. Each supernatant was tested in duplicate.

From infected CT/ST cultures, a second set of experiments was done using only the supernatants. These cells were treated with human Intravenous Immunoglobulin (IVIg; Bayer Inc., Healthcare Division, Toronto, ON) after infection to neutralize any virus remaining from the innoculum ensuring that culture supernatants were free of innoculum contamination. Cultures were prepared as previously described (section 2.1.3). CT and ST were infected with 100 µl/well of RV (1.6×10^7 PFU/ml) and cultured for 24 hours. The innoculum was removed, the cells gently washed with RT PBS, 100 µl of fresh medium and 50 µl of IVIg (50 mg/ml) added to each well and the incubation continued for another 24 hours. IVIg completely neutralized 3×10^4 PFU of RV with 100 µl of 50 mg/ml IVIg. Vero cells with virus plus 50 mg/ml of IVIg added showed that 99.8+/- 0.15% of the cells were uninfected 24 hours pi, while Vero cells infected with virus alone added showed ≥90% of the cells were infected 24 hours pi. The media was changed every 2 days thereafter, with the ST media containing EGF. At days 4, 6, 8, 10, 12, 14, or 16 the supernatants containing virus were removed and stored at –70°C. These supernatants were titered on Vero cells as described in section 2.2.2.

2.4.2 Culturing Trophoblasts on Semi-Permeable Insert Membranes

To determine whether the ST were capable of basally releasing progeny RV they were cultured on insert membranes and the supernatants analysed. Confluent ST cultures

on semipermeable membranes were prepared as previously described (63). Briefly, insert membranes precoated with fibronectin (Biocoat, Becton) were soaked in IMDM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin for 1 hour prior to plating. Trophoblasts were plated at 1 x 10^5 cells/insert each on days 0, 3 and 7 of culture and incubated for 4 hours. After plating cells were gently washed with media, and incubated at 37°C in humidified 5% CO_2. The inserts were cultured in ST medium (EGF supplemented IMDM) and the media was changed every 2 days.

As an indication of culture confluence and integrity, the transepithelial resistance (TER) of the insert was measured(63). The TER was measured with an Endohm tissue resistance measurement chamber (World Precision Instruments, Inc.; Sarasota, FL, USA), consisting of a bottom chamber and cap containing opposing circular disc electrodes. The insert was rinsed with PBS, allowed to equilibrate to RT in PBS and placed in the bottom chamber containing PBS. The Endohm cap was placed inside the insert, centering it on the bottom chamber electrode and the electrical resistance was monitored on a Millicell-ERS meter (Millipore/Continental Water Systems, Bedford, MA, USA). To obtain values independent of the membrane area, TER measurements were multiplied by the effective membrane diameter (0.3 cm^2) and reported as ohms x cm^2. The TER of the inserts was measured on day 12 to ensure a high TER of >75 ohms cm^2. Once the cultures achieved high TER the culture inserts were infected with 100 μl RV (2 x 10^6 PFU/ml) for 24 hours in ST medium. Then the inserts were gently washed with RT PBS once and fresh media containing EGF added.

2.4.3 Testing Supernatants from Membrane Cultures for Release of RV

To test for basal release of virus, two methods were used, one where the supernatants were frozen and later tested on Vero cells, and another where the Vero cells were cultured simultaneously and supernatants tested directly. Direct testing of basal and apical supernatants ensured that no virus released into the supernatant was destroyed during freezing.

In the first method, the supernatants from the insert (apical) and lower chamber (basal) were collected at various days pi and stored at -70°C. Supernatant samples were thawed and added to Vero cells without dilution at 50 µl per well. The presence of RV was determined using IH as described in section 2.2.2, and the number of RC were counted per well.

For the second method, Vero cells were plated in a 96 well plate at 1 x 10^4 cells/well and in a 24 well plate at 5 x 10^4 cells/well and cultured for 24 hours. Beginning on day 2 pi, all the inserts, both uninfected ST and infected ST, were placed in the 24 well plate containing Vero cells for 24 hours. After each 24-hour period the inserts were transferred to a new 24 well plate containing Vero cells. At the end of each 24-hour period the apical supernatants from the inserts were transferred to the 96 well plate containing Vero cells. The 24 well plate was fixed 24 hours after the inserts were removed and the 96 well plate fixed 24 hours after the apical supernatants were added. The Vero cells were fixed and stained according to methods described in section 2.2.2, and the number of RC per well was counted.

2.4.3 Measuring Membrane Integrity with Fluorescein Isothiocyanate-dextran Diffusion Following RV Infection

To monitor the integrity of the trophoblast membrane cultures following infection, diffusion of Fluorescein Isothiocyanate-dextran (FITC-dextran; Sigma Chemical Co., St. Louis, MO) across the insert membrane was measured at different times pi. 10 μg/well of FITC-dextran in 200 μl of phenol red-free IMDM (Gibco BRL, Grand Island, NY) containing 2% FBS was added to each insert for 2 hours. The bottom chambers contained 800 μl of the 2% FBS phenol red-free IMDM. Each experiment consisted of: two infected cultures grown on inserts, two uninfected cultures grown on inserts, an insert without cells to measure maximum diffusion, one bottom chamber containing only medium as a blank reference, and one bottom chamber containing 10 μg of FITC-dextran as a maximum reference. Diffusion was allowed to proceed for 2 hours, then the inserts were removed and fluorescence was measured in a fluorescence plate reader (485-530 ηm; CytoFluor 2350, Fluorescence Measurement System, Millipore; CytoFluor 2300-series System, version 3B1, Astroscan Ltd. ©1989/92).

2.5 Loss of Trophoblasts during Infection

2.5.1 Neutral Red Uptake Assay

Neutral Red was used to examine cell viability during RV infection. Neutral Red analysis was carried out as described (14). CT and ST were cultured and infected with 100 μl/well of RV (1.7×10^7 PFU/ml). At days 2, 4, 6, 8, 10, or 12 pi 50 μl of PBS containing 17.3mM Neutral Red (Sigma Chemical Co., St. Louis, MO) was added to each well, and incubated for 2 hours at 37°C in humidified 5% CO_2. Then the cells were

gently washed 3x with 200 µl of PBS, and 100 µl of 50 mM NaH_2PO_4 containing 50% ethanol was added to each well. The plate was left at RT for 5 minutes and then the absorbency determined on a plate reader at 570 – 650 ηm (Molecular Devices, Menlo Park, CA).

2.5.2 Counting Cell Nuclei and Quantifying Cell Loss

To obtain an estimate of the number of nuclei per well and the number of nuclei being lost over time, the nuclei were stained with hematoxylin as described in section 2.3. The total nuclei per well are calculated for each set of conditions, triplicate samples averaged, and numbers from infected and uninfected cultures compared to determine if relative cell loss occurred.

2.6 The Amount of Apoptosis during Infection

2.6.1 TUNEL (TdT-mediated dUTP-biotin nick end labelling)

To determine the number of apoptotic nuclei per well during infection the TUNEL assay was preformed (54, 165). CT and ST were cultured and infected with 100 µl/well of RV (1.6 X 10^7 PFU/ml). On days 2, 4, 6, 8, 10, or 12 pi the cells were fixed with ice cold methanol: acetone (1:1) for 10 minutes at 4°C. The cells were washed 2x with PBS and stored in PBS at 4°C in the dark. 100 µl of Terminal deoxynucleotidyl transferase (TdT) buffer containing 30.0 mM Trizma base, pH 7.2, 1.0 mM cobalt chloride, and 139.7 mM sodium cacodylate was added to each well for 10 minutes at RT. 30 µl of TdT reaction mixture containing: 5 µl TdT buffer, 1 ηM Bio-UTP (Roche Diagnostics, Mannheim, Germany), 9.925 U TdT enzyme (Roche Diagnostics, Mannheim, Germany), 1.36 µM dATP (Boehringer Mannheim, Laval Quebec) was prepared and added to each

well and incubated at 37°C for 1 hour. The reaction was terminated with 100 µl per well of 2xSSC buffer containing 600 mM sodium chloride and 60 mM sodium citrate. The cells were washed 3x with PBS.

The cells were stained for TUNEL positive nuclei and RV using indirect immuno-fluorescence with fluorescent-tagged secondary antibodies. 50 µl per well of 3% H_2O_2 was added for 5-10 minutes, the cells were washed 3x with PBS, and then 75 µl per well of 3% skimmed milk in PBS/0.5% Tween 20 (Anachemia Chemicals Ltd., Toronto, ON) added for 30 minutes at RT. Then 50 µl per well of primary antibody, either a mouse monoclonal to RV E1 glycoprotein at 5 µg/ml or its IgG1 isotype control at 5 µg/ml, added for 30 minutes. The primary antibody was removed and the cells washed 4x with PBS. Then 50 µl per well of goat anti-mouse IgG texas red-conjugated at 1 µg/ml Alexa Fluor 546 (Molecular Probes, Eugene, Oregon) and FITC-conjugated streptavidin at 1 µg/ml Alexa Fluor 488 (Molecular Probes, Eugene, Oregon) in PBS containing 3% skim milk and 0.5% Tween 20 was added for 30 minutes at RT. The cells were washed 3x with PBS and stored at 4°C in PBS.

To visualize all nuclei, 100 µl of PBS containing 1.4µg/ml DAPI (4,6-diamidino-2-phenylindole) (Molecular Probes, Eugene, Oregon) was added to each well for 10 minutes at RT. Cells were then washed 3x with PBS and stored in PBS at 4°C. To analyse the TUNEL positive nuclei (Alexa Fluor 488; FITC), E1 positive cells (Alexa Fluor 546; texas red) and the total nuclei (DAPI), computer imaging was used. The cells were visualized with an inverted phase contrast microscope (Model DS-IRB, Lecia; Heerbrugg, Switzerland) equipped for epifluorescence with a 100W high-pressure mercury lamp driven by a Ludl power source (Ludl Electronic Products; Hawthorne,

NY). Digital images were taken of each well using a Spot digital camera (Diagnostic Instrument; St Sterling Height, Michigan) using Spot Advanced program (version 2.2.1, Diagnostic Instruments Inc., ©1997-1999). The same 5 areas in each well were photographed for total nuclei, TUNEL, and E1 staining. Quantification analysis was done using Image-Pro Plus (version 4.1.0.0 for Windows 95/NT/98, Media Cybernetics, L.P., ©1993-1999; Del Mar, CA). Calculations were then made to approximate the percentage of TUNEL positive nuclei per well, the ratio of RC to nuclei, and the total nuclei per well. These numbers were calculated for each set of conditions, duplicate samples averaged, and numbers compared to determine if apoptosis increased with increase in cell loss.

2.6.2 Inhibiting Apoptosis

To look at the role apoptosis plays in the cell death of trophoblasts, two apoptotic inhibitors, Z-VAD-FMK and anti-TNFα, were examined during infection. ST and CT cultures were prepared as previously described (section 2.1.3), except the trophoblasts were cultured in IMDM/2% FBS after the 4-hour adherence time. One hour prior to infection 100 μM Z-VAD-FMK (Kamiya Biomedical Company, Seattle, WA) or carrier control (DMSO) was added to the cultures. Simultaneous with infection 20 μg/ml of polyclonal anti-human TNFα antibody (anti-TNFα, ICN, Aurora, OH) was added to the cultures. The cultures were infected with RV (1.6×10^6 PFU/well) and incubated for 24 hours at 37°C in humidified 5% CO_2. The virus was removed and media added with fresh inhibitor or carrier added. The media was changed every 24 hours with new inhibitor or carrier added each time. At a predetermined day pi the cells were fixed,

TUNEL assay done, immunofluorescent staining carried out, and digital analysis preformed as previously described in section 2.6.1.

Prior to this inhibition experiment with Z-VAD-FMK, a control experiment was done to ensure 100 μM (the recommended dose) was the optimal concentration for inhibition of apoptosis. For this experiment, CT were stimulated with 10 ηg/ml recombinant human TNFα (a gift from Hofmann La Roche, Basel, Switzerland) and 100 units/ml IFNγ (Becton Dickinson) to induce apoptosis and Z-VAD-FMK added at 200, 100, 50, and 25 μM 1 hour prior to TNFα/IFNγ addition. Results indicated that 100 μM was the concentration of Z-VAD-FMK that yielded the lowest percentage of apoptosis (0.45 +/- 0.35 %) and the highest total nuclei per well (42272 +/-1358 nuclei). TNFα/IFNγ without Z-VAD-FMK added induced 7.9 +/-1.3 % apoptosis and had 37376 +/-724 nuclei after 24 hours.

2.6.3 Determining the Effect of EGF on Apoptosis and Cell Loss

To examine the effect of EGF on trophoblasts, ST and CT were cultured with and without EGF in both uninfected and infected cultures. ST and CT cultures were prepared as previously described (section 2.1.3). Simultaneous with infection EGF (10ηg/ml) was added to the CT media for one infected and one uninfected CT culture, but EGF remained absent from the other CT cultures. Similarly, at the same time as infection, one infected ST culture and one uninfected ST culture had no EGF present in the media, but the remaining controls and infected cultures had regular ST media. The cultures were infected with 1.6×10^6 PFU/well of RV and incubated for 24 hours at 37°C in humidified 5% CO_2. Virus-containing media was removed, fresh media added then and every 24-

hours, with fresh EGF added to cultures as previously stated. At a predetermined day pi the cells were fixed with methonal:acetone (1:1), TUNEL assay and immunofluorescent staining carried out, and digital analysis preformed as previously described (section 2.6.1).

2.7 Infection and Apoptosis in the Non-Adherent Population

2.7.1 Determining Apoptosis and Infection in the Non-Adherent Cell Population

To determine whether lost trophoblasts were infected or apoptotic, the non-adherent population was retrieved and examined for RV and TUNEL positive nuclei. At days 4, 6, 7, 8, and 9 pi the supernatants were collected and spun onto glass microscope slides (Mercedes Medical, Sarasota, Florida) using a cytospin (Cytospin 2, Shandon, Johns Scientific) at 500 rpm for 5 minutes. The slides were fixed in methanol:acetone (1:1) for 10 minutes at 4°C then placed in PBS for 10 minutes at RT. TUNEL assay and indirect immunofluorescent staining for E1 were carried out on the slides immediately and they were stored in PBS at 4°C in the dark (Methods section 2.5.1). Photomicrographs were taken and digital analysis performed. Digital images were taken of each slide; five areas were photographed for total nuclei, TUNEL, and E1 staining. Calculations were then made to approximate the ratio of TUNEL positive nuclei, the ratio of RC-associated nuclei, the ratio of TUNEL positive and RC-associated nuclei, and the ratio of TUNEL negative and uninfected nuclei per slide.

2.8 Statistical Analysis

Experiments for each figure were performed two or three times (as stated in each figure) on trophoblasts from different placental preparations. Differences between experimental groups were evaluated by one-way ANOVA (analysis of variance) with pairwise multiple comparison procedures (Tukey Test) using SigmaStat program (Jandel Scientific, San Rafael, CA). Results were considered to be significant at $p<0.05$.

CHAPTER 3: RESULTS

3.1 The Infection of Trophoblasts

One of the first questions I addressed was whether or not primary trophoblasts could be infected with RV. Previous pathology studies indicate RV infects the villous trophoblast and suggests RV passes to the fetus through the villous placenta (141, 150). One hypothesis is that RV reaches the fetus by passing through the villous trophoblasts in the placental barrier. It is important to clearly establish the conditions under which trophoblasts can be infected with RV and the conditions quantifying this infection.

First, steps were taken to ensure IH was a valid method for titering RV and could be used to quantify infection in trophoblasts. Thus, IH was compared to plaque formation, an accepted method for quantifying RV infection (89). The IH method, adapted from Hobbins and Smith (65), stained specifically for the E1 glycoprotein of RV. Both methods showed results that could be repeated and gave titers comparable to one another (Figure 5). Henceforth, RC (Vero IH) is equated to PFU (plaque assay). To determine if RV infects trophoblasts, CT and ST were infected with RV and stained for E1 glycoprotein using IH. Since RV titers determined by IH in Vero cells paralled those determined by the plaque-forming assay in RK13 cells, the same criteria were used to quantify the amount of infection in trophoblast cultures. According to the IH staining of the E1 glycoprotein, both CT and ST cultures could be infected with RV. Forty-eight hours after infection with $1x10^6$ PFU of virus, both CT and ST contained discrete spots of intense E1 glycoprotein staining (Figure 6), suggesting infection foci or RC. The amount of infection is expressed as RC per nuclei rather than percent infected. Trophoblasts

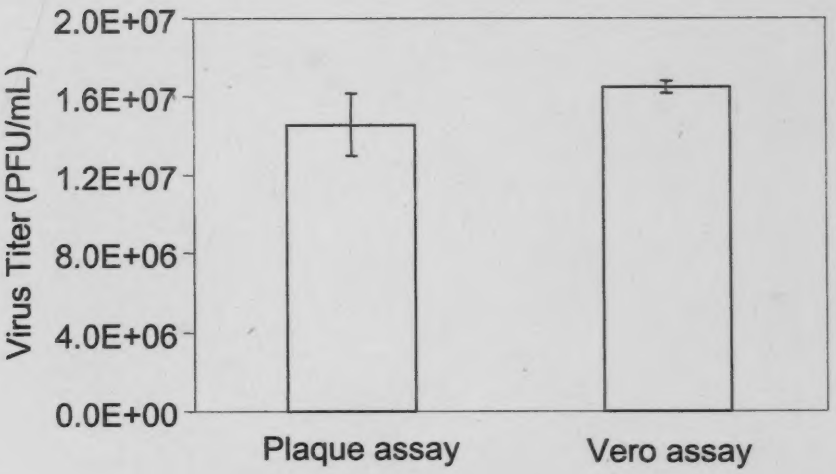


Figure 5. Comparing two titer methods - plaque assay and Vero immunohistochemistry - used to titer Rubella virus. The results are obtained from titering the virus stock RV 09.09.00 at 10^6 dilution. The plaque assay was repeated three times and the Vero assay two times. The error bars represent the standard deviation.

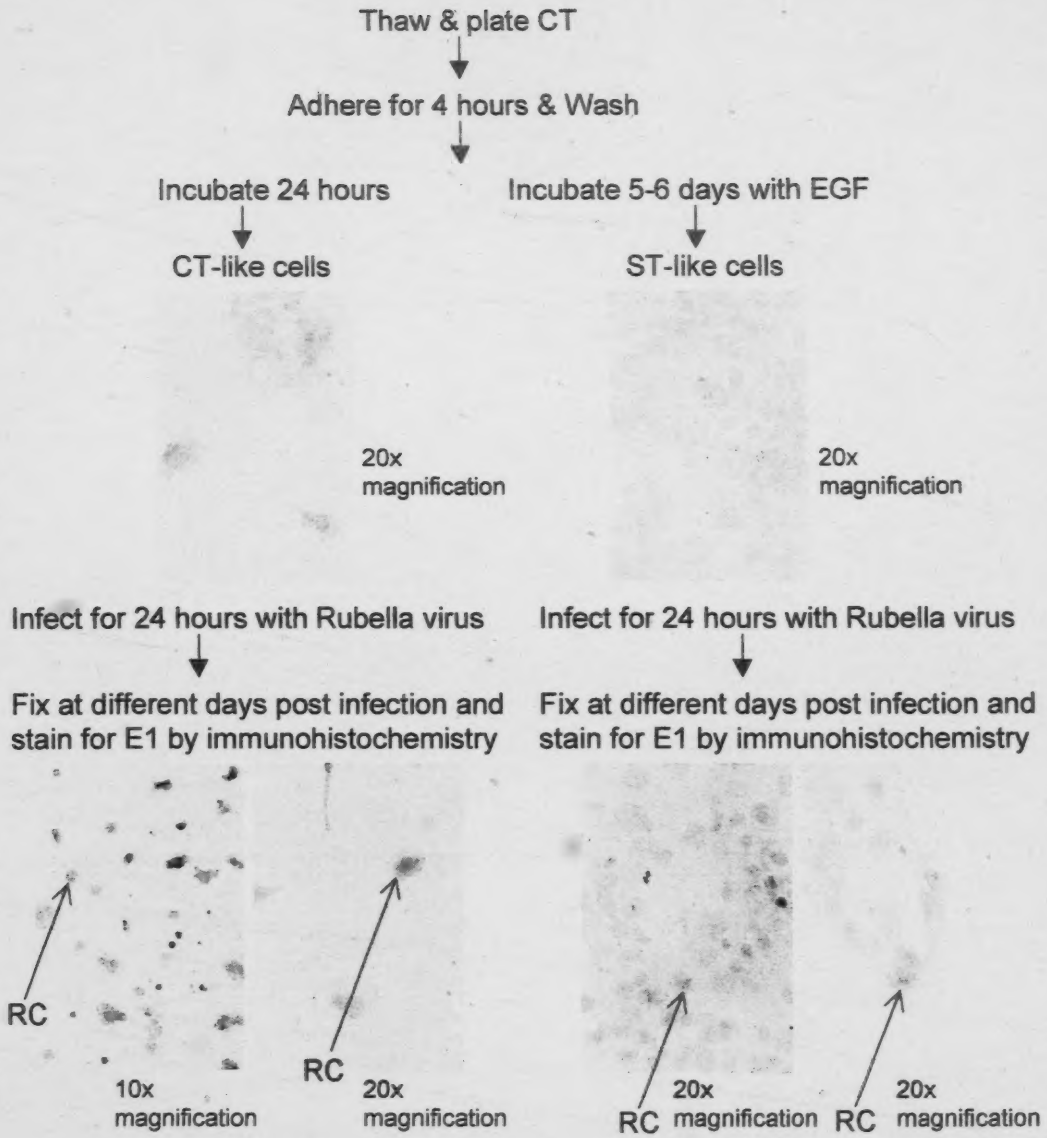


Figure 6. Time course for culturing and infecting CT and ST. Photomicrographs indicate uninfected CT and ST, and infected CT and ST at day 2 post infection. RC (replication center) indicates positive E1 staining indicating Rubella virus infection.

form multinucleated cells, and for this reason, expressing infection as a percentage of cells would be misleading and more difficult to interpret.

In order to detect infection in trophoblasts in vitro, a relatively high titre of virus was needed compared to that for the control cell line Vero. CT, ST, and Vero cells were cultured, infected with RV, and stained for infection using IH. Figure 7 shows the dose response of CT, ST, and Vero infection 2 days after challenge. As well, the level of infection at these higher doses was much lower in trophoblasts than in Vero cells. The ratio of RC to nuclei was 1.00 in Vero cells, compared to 0.14 in CT at $1x10^4$ PFU of virus added. Similarly the ratio of RC to nuclei was 0.02 in ST, while it was 1.00 in Vero cells at $4x10^4$ PFU of virus. These results demonstrate that Vero cells were more efficiently infected with RV than CT, and CT was more efficiently infected than ST.

Next, I examined the amount of infection as a function of time (Figure 8). The infection was monitored over 8 days in both CT and ST cultures. Infection in CT cultures either continuously increased or reached a maximum within 4 days. In contrast, in ST cultures, the infection was maximal by day 2 pi and thereafter decreased. Again, a higher amount of infection was detected throughout the experiment in the CT, ranging from a ratio of 0.02-0.12 RC to nuclei, compared to the ST, which stayed below a ratio of 0.02 RC to nuclei. The exact trend of infection varied with each placental preparation, but it is important to note that infection remained throughout the assay in all preparations.

3.2 Trophoblasts Infection is Productive

Once the methodology for infection and IH were established and it was confirmed that the trophoblasts could be infected with RV, I next sought to determine whether or not infection of CT and ST was productive. In my hypothesis I stated that the RV possibly

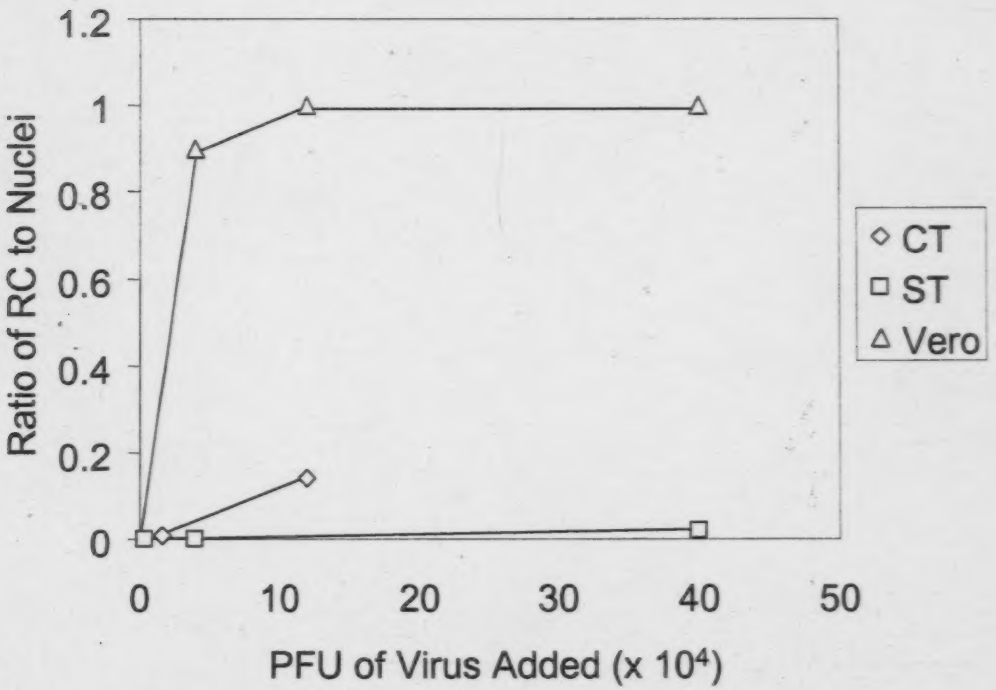


Figure 7. Infection of Vero cells, CT, and ST as a function of Rubella virus dose. Different amounts of virus were added as indicated, and the cells fixed and stained for E1 glycoprotein at 2 days pi. The amount of infection was calculated as a ratio of replication centers (RC) to the number of nuclei. These results are from one experiment, but the experiment was repeated once with similar results.

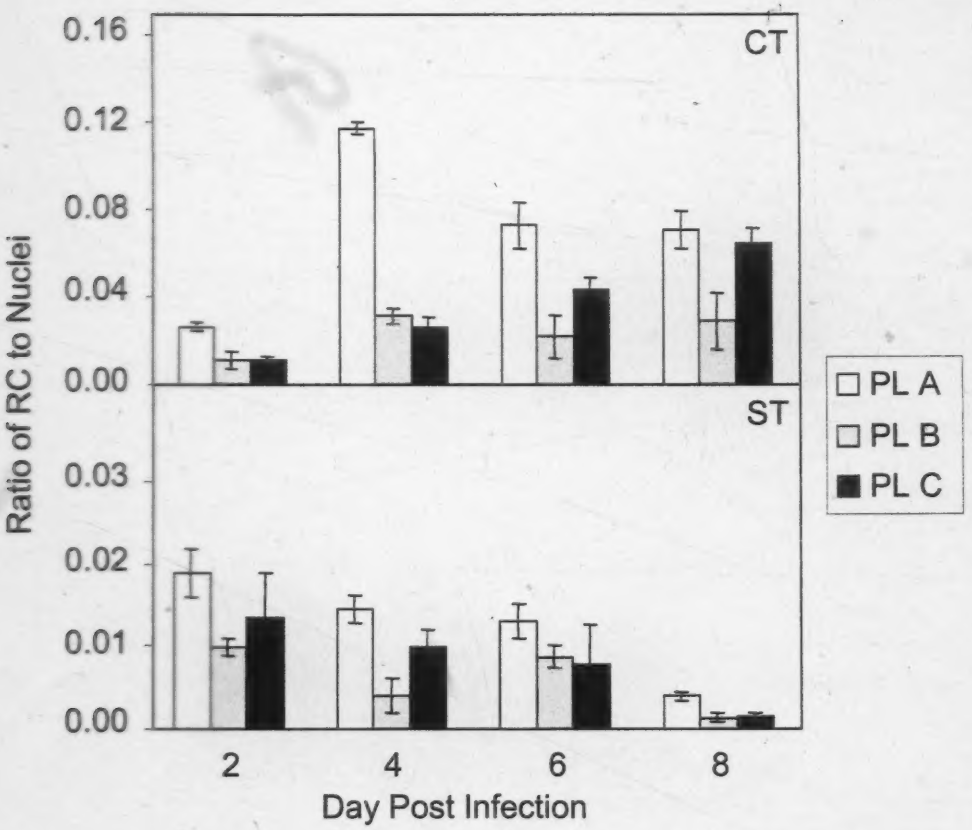


Figure 8. The amount of infection in CT and ST over time expressed as the ratio of replication centers (RC) to the number of nuclei. CT and ST were cultured, infected with $1.6x10^6$ PFU/well of RV and carried out over 8 days post infection for both CT and ST. The cultures were fixed with methanol and stained using immunohistochemistry for RV. The bars depict the average +/- SD obtained from triplicate wells. The data above depict three experiments, each experiment using a different placental preparation.

reached the fetus through directional release of RV basally from the ST. Establishing virus production and release would be key to looking at any basal release of the virus from the ST.

Initially, these experiments were performed by comparing the amount of virus released into the supernatant to the amount that was retained inside the cell. The supernatants and cellular lysates were collected and tested for the presence of RV by infecting Vero cells and determining the presence of infection by IH. This experiment was performed twice, using different methods of cellular lysis – freeze thawing and sonication – and both yielded similar results. The majority of the virus (more than 70%) was present in the supernatant and very little was found associated with the cells (Figure 9). Since the amount of virus produced was highest on day 2 pi, it was necessary to ensure that none of the virus recovered was contaminated with virus from the initial innoculum. It was important to make certain that the CT and ST were actually producing and releasing RV before continuing. To do this, the experiment was repeated with IVIg added to the cultures before harvest, then washed away, to neutralize all extracellular virus remaining (see Methods section 2.4.1). This ensured that none of the samples had virus left from the initial virus challenge. This experiment was carried out on three different placental preparations, and only the supernatants were collected since the previous experiment demonstrated that the majority of the virus was in the supernatant. Again, both the CT and ST produced and released RV able to infect Vero cells (Figure 10). It is important to note that the data represent the steady state amount of infectious virus that was in the supernatants of infected trophoblast cultures that underwent media changes every two days. The amount of virus detected in the supernatants of CT

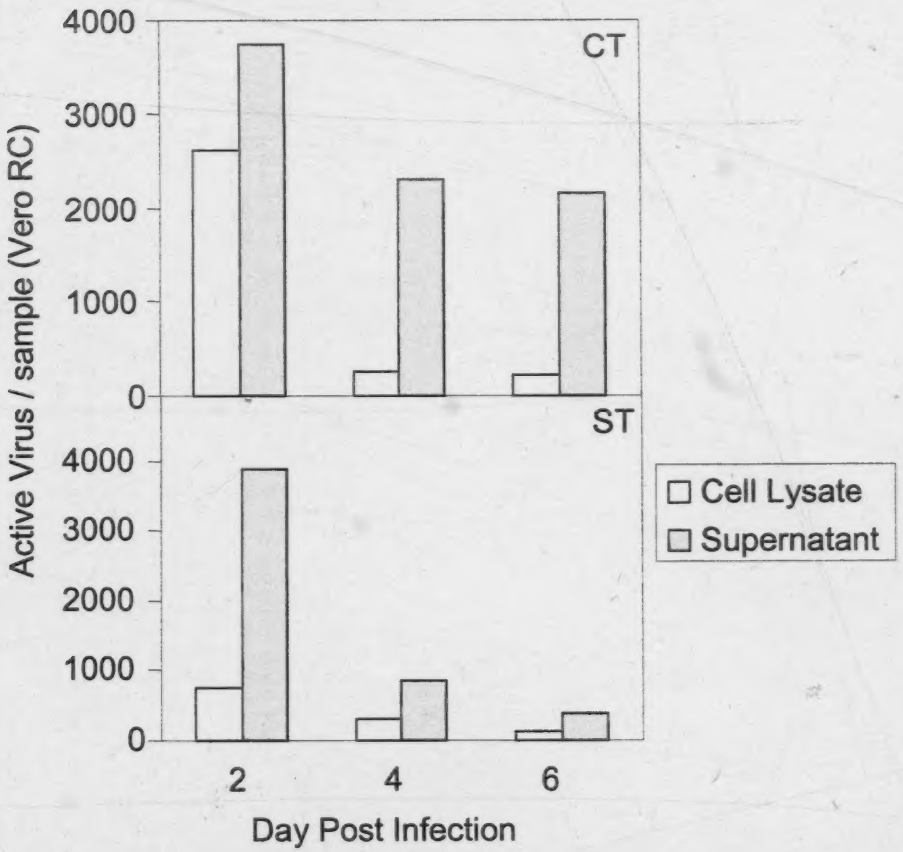


Figure 9. Rubella progeny virus production from trophoblast supernatants and cellular lysate. Supernatant from trophoblast cultures were collected pi, and the cells lysed by sonication. The samples were frozen and later tested on Vero cells. The Vero cells were infected with samples, fixed on day 1 pi and stained using immunohistochemistry for E1 glycoprotein. The number of RC were counted per well. This experiment examined single wells but was repeated once with similar results.

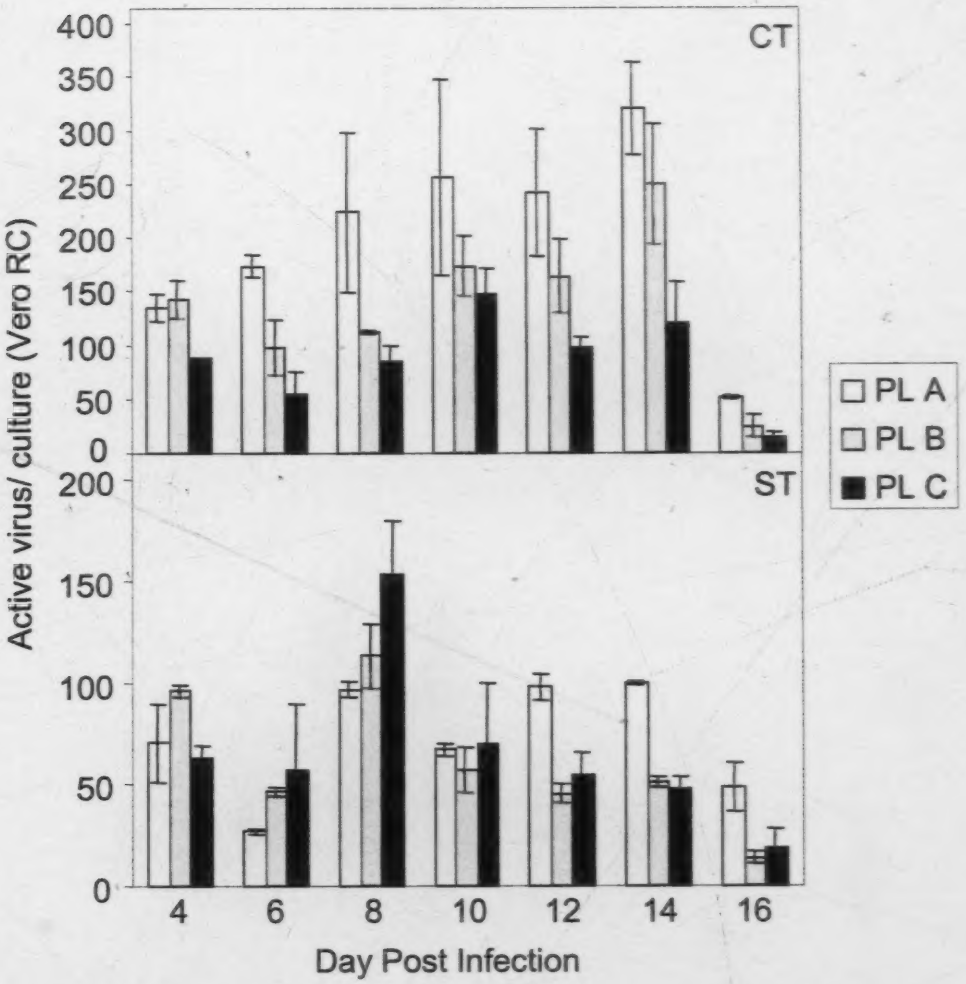


Figure 10. Active virus production from trophoblasts treated with IVIg after infection. Both CT and ST supernatants were collected from three experiments, each experiment used a different placental preparation. The cultures were infected for 24 hours with $1.6x10^6$ PFU/well of RV, the virus was removed, and then the cultures were treated with IVIg for 24 hours. The supernatants were collected from days 4 to 16 pi, and tested on Vero cells for the presence of RV using immunohistochemistry for E1 glycoprotein. The error bars represent the range of results from duplicate wells.

indicated a peak virus release at around days 10-14 pi. The ST show peak virus release after day 8 pi, depending on the placental preparation. It is important to keep in mind that the virus detected may be released as a result of cellular lysis or loss of adherence due to infection and not active secretion from the trophoblasts. The amount of virus production varied with each placental preparation, but remained much lower than virus production in the cell line Vero. Vero cells produce 10^3-10^5 PFU/mm^2 of confluent culture, whereas the trophoblasts produced only 1-10 PFU/mm^2 of confluent culture. The data indicate that trophoblasts are capable of producing and releasing active RV, and thus further investigation is warranted into whether or not this release is directional.

3.3 Directional Release of RV from Trophoblasts

One possible mechanism of reaching the fetus is through basal secretion from the ST. ST were cultured on membrane inserts, and both apical and basal release of virus was analysed as described in the Methods section 2.4.3.

In order to determine if the virus release into the lower compartment was due to basal secretion, it was important to monitor the integrity of the cultures. Any cell loss or loss of membrane integrity could result in non-specific passage of virus through the damaged membrane culture. FITC-dextran diffusion (average molecular weight: 2,000,000) was used as an indicator of membrane integrity. The integrity of the cell layers was monitored by measuring the amount of FITC-dextran diffusion in one hour at various days pi and simultaneously measuring virus release into the upper (apical) and lower (basal) chambers of parallel cultures. The results in Figure 11 show loss of membrane integrity in infected but not control cultures by day 5 of infection. The experiment was repeated with a different placental preparation (Figure 12). This time, even control

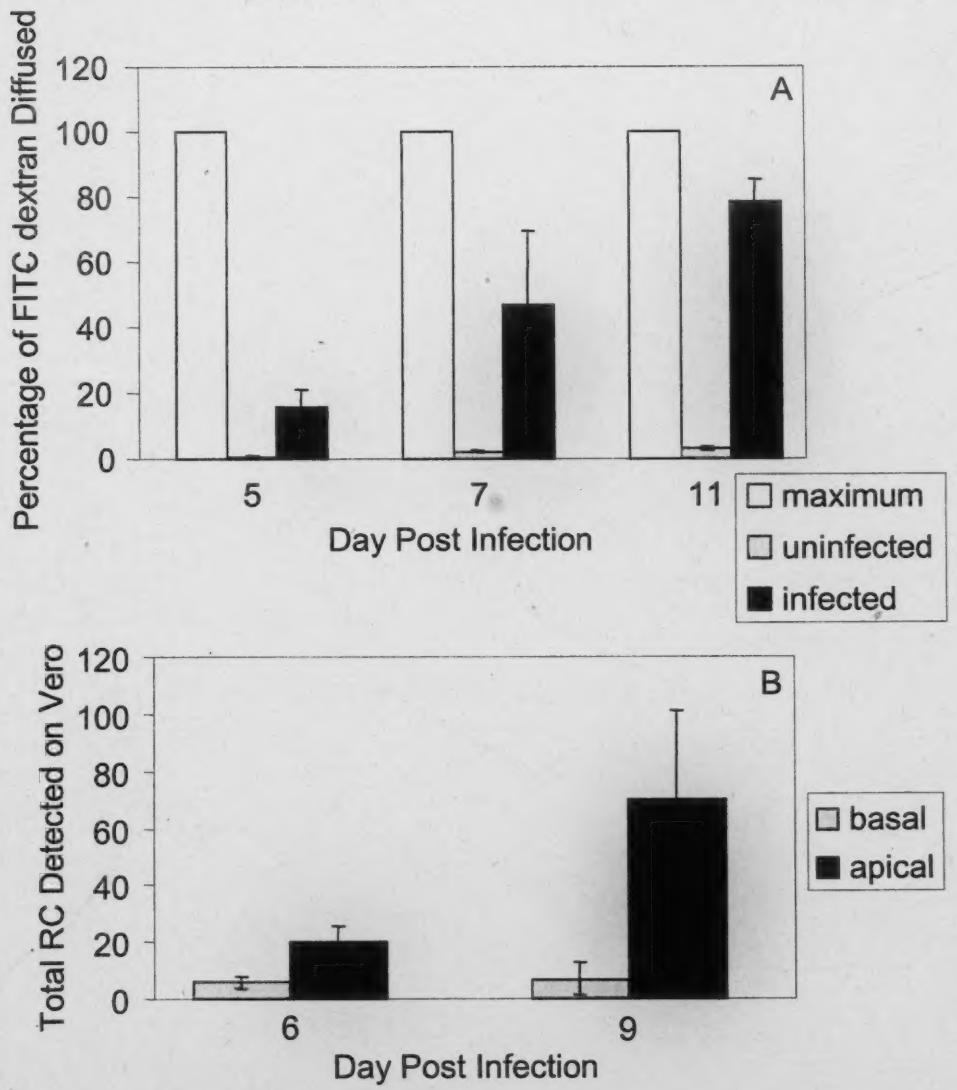


Figure 11. The basal release of Rubella virus from ST late in infection with FITC –dextran diffusion as an indication of membrane integrity. ST were plated on membrane inserts, infected with $2x10^5$ PFU/well of Rubella virus and the membrane integrity monitored with FITC –dextran diffusion. **A.** The membrane integrity is monitored by measuring the amount of FITC dextran that can diffuse within one hour. A blank insert is used as the maximum control. The bars of uninfected and infected cultures represent duplicate wells with error bars representing the standard deviation. **B.** ST were cultured on membrane inserts, apical and basal supernatants collected pi, and tested on Vero cells. Vero cells were fixed 1 day post infection and stained with immunohistochemistry for E1 glycoprotein. The results are from duplicate wells with the error bars representing the standard deviation.

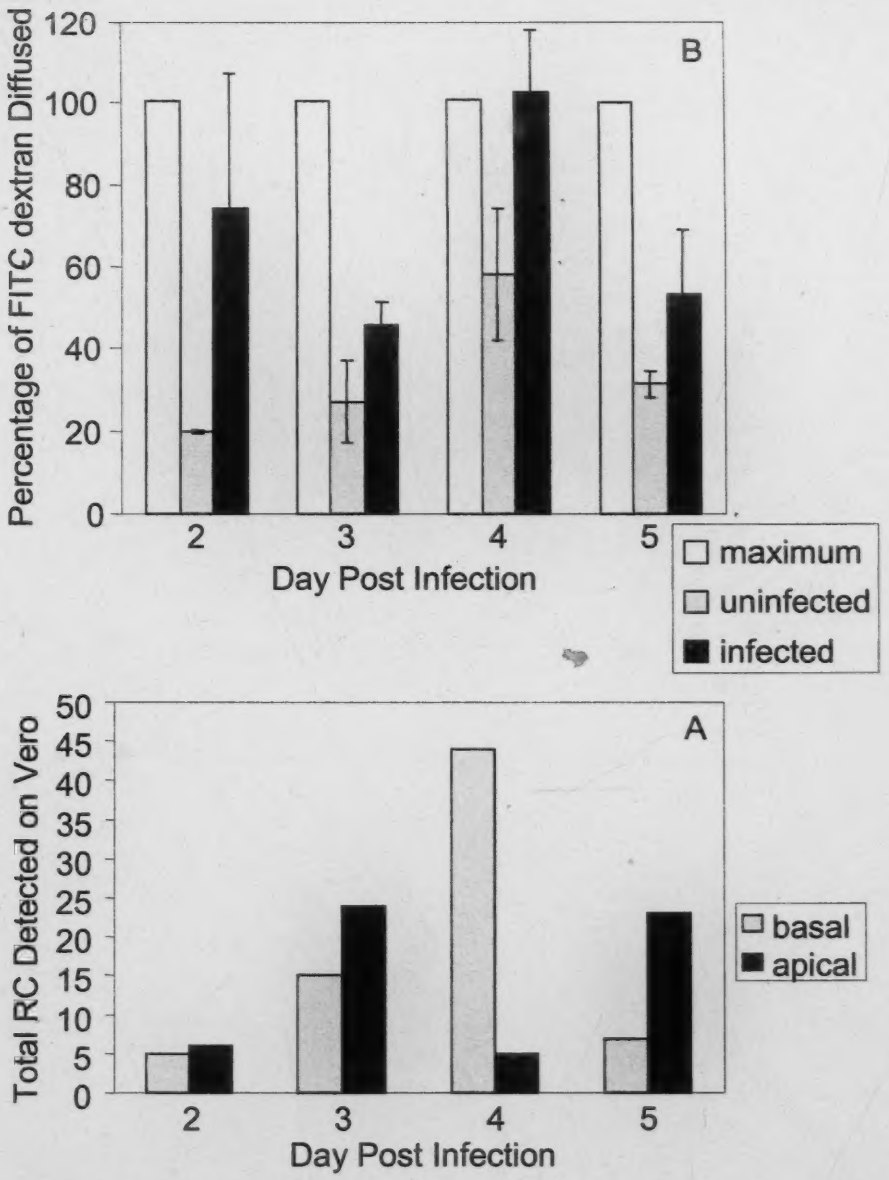


Figure 12. Basal release of Rubella virus from ST at an early time point during infection. ST were plated on membrane inserts, infected with $2x10^5$ PFU/well of Rubella virus and the membrane integrity monitored with FITC –dextran diffusion. **A.** The FITC –dextran diffused for 1 hour and a blank insert was used as a maximum control. The bars represent duplicate wells with the error bars representing the standard deviation. **B.** The apical and basal supernatants were directly tested on Vero cells, and the cells fixed and stained using immunohistochemistry for E1 glycoprotein. The bars represent one well.

cultures had lost membrane integrity by day 2 of culture, but infection increased FITC-dextran diffusion at all time points. Thus, generally, the infected cell layers began to deteriorate at an earlier time point and continued to deteriorate more rapidly over time in comparison to the uninfected cell layers. However, we found that relative membrane integrity is highly dependant on the placental preparation.

The virus present in the apical supernatant indicated that the trophoblasts were infected and producing infectious virus (Figures 11 and 12). Figure 11, which involved freezing all supernatants, revealed that little active virus was present in the basal supernatant. Since freezing can reduce the amount of virus, another experiment was performed directly testing the supernatants on Vero cells. Figure 12 indicated that considerable virus was reaching the basal chamber when the membrane integrity was poor. While these results do not provide evidence for polarized release of RV from infected ST, they do provide evidence that infection occurs on the apical surface of ST and that such infection leads to rapid loss of ST culture integrity.

3.4 Loss of Trophoblasts during RV Infection

Since this project focused on the passage of virus across a barrier, it is important to note the effects of infection, specifically any cell loss that occurs during infection. The basal release experiments indicated that RV caused loss of trophoblast integrity, possibly due to loss of cells. RV is known for its cytopathic effect (CPE) on some cell lines (47, 48, 129). Therefore, we more closely examined whether RV could induce CPE in trophoblasts. This was studied in three ways detailed below.

3.4.1 Neutral Red Assay

Neutral Red assay is a method often used to look at cell viability. Neutral Red is a dye that is taken up by viable cells and later extracted and measured by optical density. Neutral Red data indicated that a significant amount of cell loss occurs during RV infection. Comparing the uninfected cultures of CT to the infected cultures of CT as seen in Figure 13, there was about 50% cell loss after day 4 pi. This cell loss increased over time in the CT cultures. Similar results can be seen with the ST cultures, with about 40% cell loss 6 days after infection (Figure 13). Again, the cell loss continued over time in the ST but at a much slower rate compared to the CT. The results were the average of three experiments, each with trophoblasts from a different placenta. Visual examination of the cultures, however, clearly indicated few live cells remaining late in the ST culture even though the Neutral Red assay indicated ~ 50% loss. This comparison indicated that the Neutral Red assay was not linear. Still, the results from the Neutral Red assay indicated that a significant amount of virus-induced cell loss was occurring; therefore more quantitative analysis was required.

3.4.2 Quantifying Loss of Trophoblast Nuclei

Visual examination indicated that RV was inducing substantial cell loss during trophoblast culture. In order to document the obvious differences between Neutral Red detection of cell loss and the actual number of cells remaining after virus infection, infected and uninfected cultures were stained with the fluorescence nuclear dye DAPI. The cultures were then photomicrographed day 4 pi and day 9 pi (Figure 14). These photomicrographs confirmed that more cell loss was occurring than was detected by Neutral Red uptake and suggested a more quantitative measure of cell loss: nuclei count.

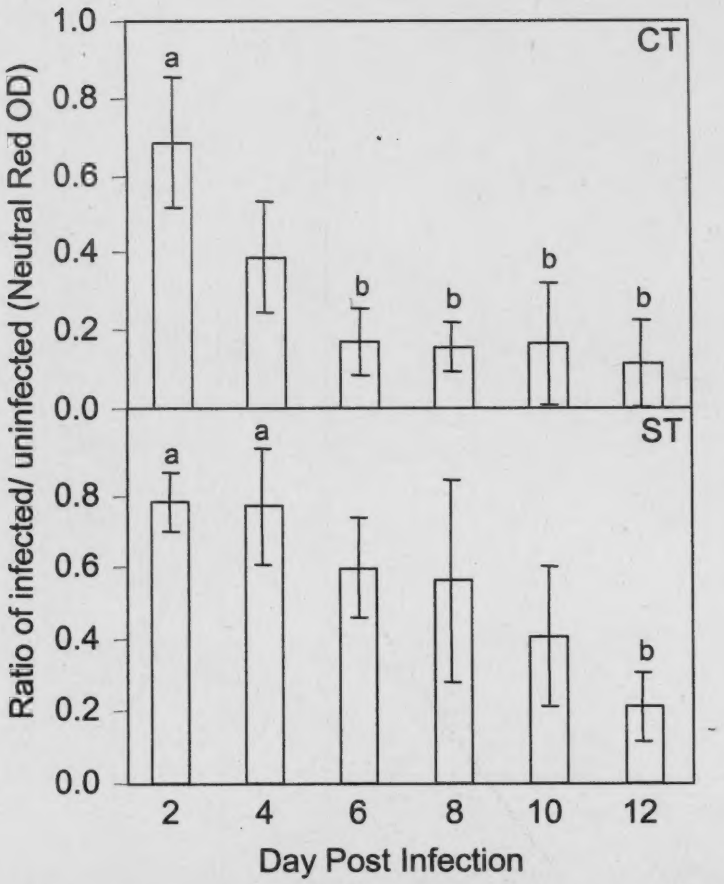
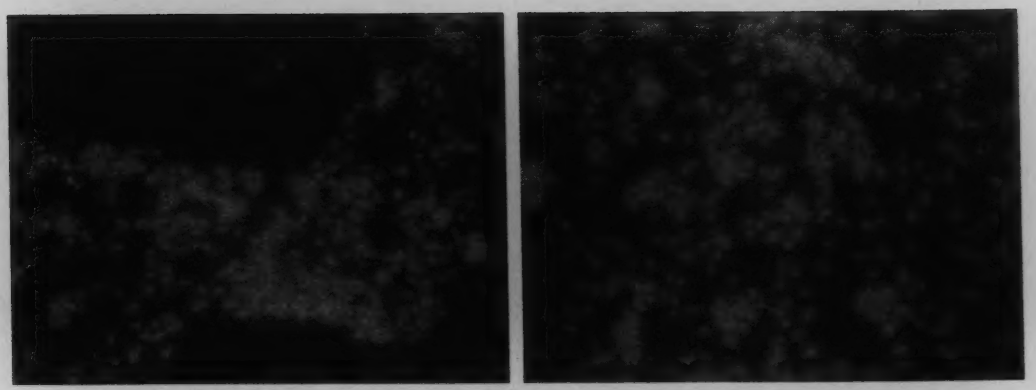
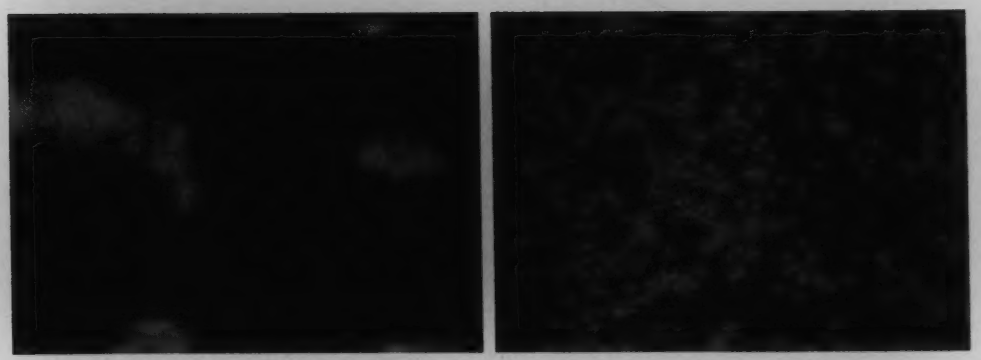


Figure 13. Ratio of the Neutral Red OD readings of infected cultures to uninfected cultures for both CT and ST as an indication of cell viability. CT and ST were infected with $1.6x10^6$ PFU/well of RV and cultured over 12 days. On each day pi cultures were treated with Neutral Red for 2 hours, and the OD was measured. The data are the average of ratios from three experiments, each using a different placental preparation. The error bars represent the standard deviation between experiments. (The ratios for each individual experiment are calculated from triplicate wells.) Statistically using one –way anova, the difference between a and b is significant, $p<0.05$.



Infected ST – day 4 pi Uninfected ST – day 4 pi



Infected ST – day 8 pi Uninfected ST – day 8 pi

Figure 14. The loss of total nuclei in ST cultures during infection. ST were cultured and infected with $1.6x10^6$ PFU/well of RV. On days 4 and 8 pi the cultures were fixed and stained with DAPI. The DAPI stained nuclei of the ST cultures, both uninfected and infected, are shown at days 4 and 8 pi. These are examples of one photomicrograph that was analyzed using digital analysis.

Trophoblasts form variably sized multinucleated cells through cellular fusion; thus, the relationship between nuclei loss and cell loss may not follow an exact ratio. However, by making the assumption that the relative amount of cell loss is proportional to the relative amount of nuclei loss, I can use nuclei count to quantify the amount of cells (or cellular matter) being lost. A decrease in nuclei indicates a loss of cells and a breakdown in cell viability. The number of nuclei in a grid (1 mm^2 at 10x magnification) was counted in 5 different areas of each well. Based upon the area of the whole well (33 mm^2 in a 96-well plate) the total number of nuclei per well was calculated. Both the total nuclei per well, as well as the ratio of total nuclei in infected cultures to uninfected cultures, were used to analyse the amount of cell loss seen during infection.

The data showed that the uninfected cultures of trophoblasts survived much longer than the infected cultures. Figure 15 shows the results for CT cultures, indicating that at day 4, a considerable amount of nuclei loss is seen during infection and continues until more than 90% of the nuclei are lost. Even though the number of nuclei associated with infection is low (Figures 7 and 8, ~10%), the number of nuclei lost during infection is much higher, with at least 50% of the nuclei gone by day 4 pi in CT cultures. This result suggests that not all the cells being lost are infected. Figure 16 indicates a similar result for ST cultures showing that after day 6 of infection major nuclei loss occurs. This nuclei loss continues throughout infection until all the nuclei are lost. Each figure shows the results for three different placentas, with differences in total nuclei between placental preparations, but similar decreases are seen over time within each preparation. With nuclei count as a measure of culture viability, these results show that RV infection of both CT and ST results in ongoing cell loss that continues until the whole culture is lost.

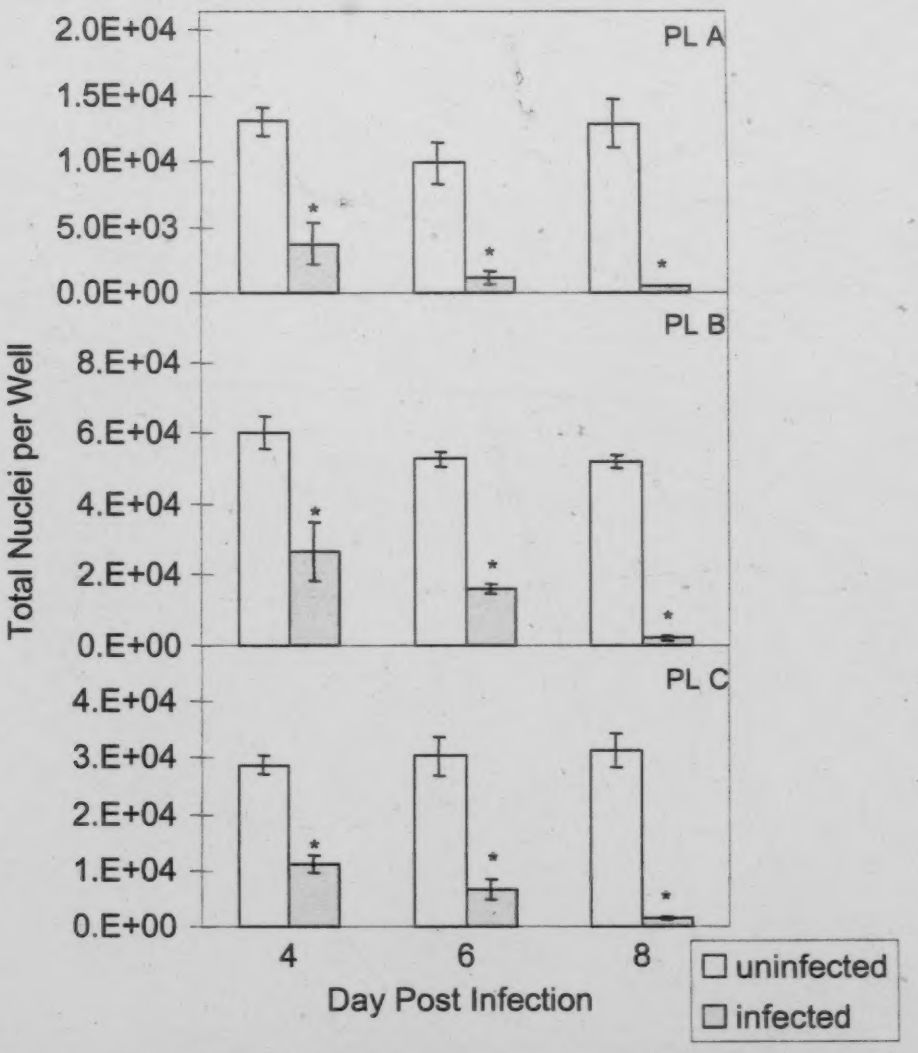


Figure 15. Cell loss from Rubella infected CT cultures determined by nuclei counts. CT were cultured and infected with $1.6x10^6$ PFU/well of RV. On the indicated day pi the cultures were fixed and stained with hematoxylin. The total nuclei per well were calculated for uninfected and infected cultures of cytotrophoblasts. The bars represent the mean +/- the standard deviation. *The total nuclei of the infected culture is significantly different than the total nuclei of the uninfected culture for each day indicated (one –way anova, $p<0.05$). This experiment was repeated three times, each with trophoblasts from a different placenta (PL A, PL B, PL C).

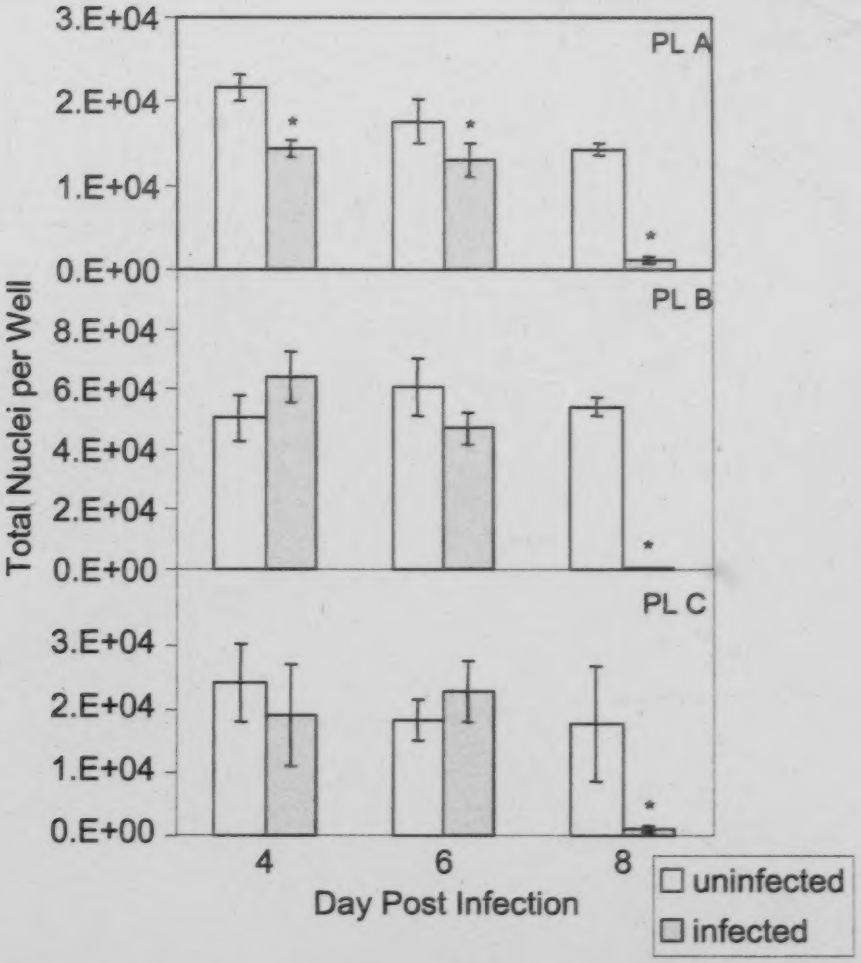


Figure 16. Cell loss from Rubella infected ST cultures determined by nuclei counts. ST were cultured and infected with $1.6x10^6$ PFU/well of RV. On the indicated day pi the cultures were fixed and stained with hematoxylin. The total nuclei per well were calculated for uninfected and infected cultures of ST. The bars represent the mean +/- the standard deviation. *The total nuclei of the infected culture is significantly different than the total nuclei of the uninfected culture for each day indicated (one –way anova, $p<0.05$). This experiment was repeated three times, each with trophoblasts from a different placenta (PL A, PL B, PL C).

3.5 The Amount of Apoptosis in Infected Trophoblast Cultures

At this point it was clear that trophoblasts were being lost during Rubella infection. RV is known to induce apoptosis in some cell lines – Vero, RK13, and BHK21 (38, 112). Trophoblasts are capable of dying via apoptosis (165) and use apoptosis as a method of removing aging cells (144). Since RV is capable of inducing apoptosis and trophoblasts are known to undergo apoptosis, the question of whether or not RV was inducing apoptosis in trophoblasts was inevitable. It was necessary to establish whether the cell loss occurring during infection was due to apoptosis or some other mechanism.

One method of quantifying the number of apoptotic cells in culture is through TUNEL, which labels the nicked DNA inside the nucleus. The amount of apoptosis in trophoblast cultures was determined using the TUNEL assay, which captures one stage of apoptosis and provides a relative approximation of the amount of apoptosis occurring. Both CT and ST were subjected to the TUNEL assay, stained using immunofluorescence and then digital analysis was performed. Figure 17 shows the total number of apoptotic nuclei detected in CT cultures, both infected and uninfected, as well as the total number of nuclei present. Panel A shows that the number of apoptotic nuclei in the infected cultures was low and not significantly different from that seen in the uninfected cultures, while the total nuclei continued to decrease throughout infection. Similar results were seen with the ST (Figure 18), the total number of apoptotic nuclei was low in infected cultures while the total number of nuclei decreased in the infected cultures over time. The decrease in total nuclei during infection is significant for CT on days 4 and 6 pi and for ST on day 8 pi. The data also indicated that the number of apoptotic nuclei in infected cultures was much less than the decrease in nuclei seen throughout infection.

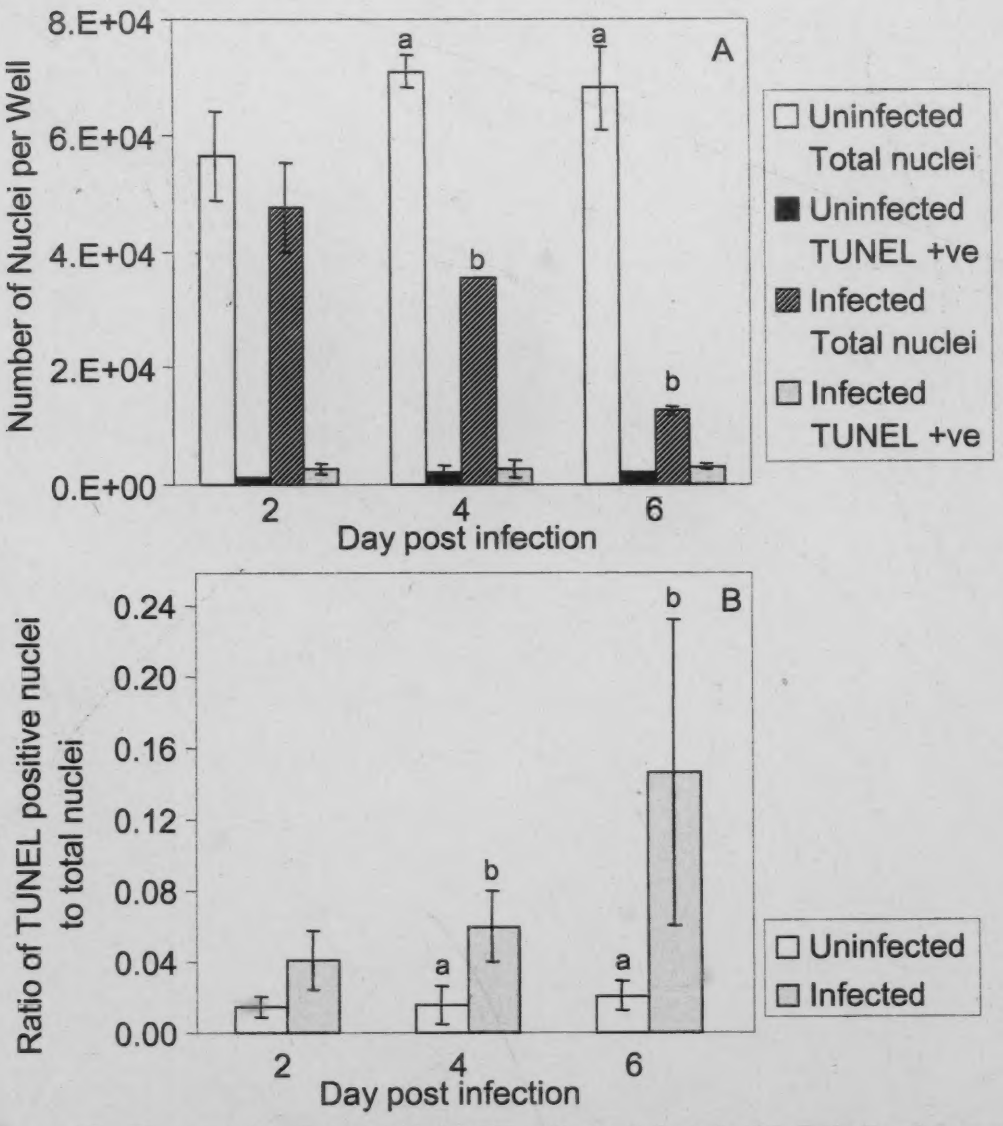


Figure 17. The amount of apoptosis in CT cultures infected with Rubella virus as detected with the TUNEL assay. CT were cultured, infected with $1.6x10^6$ PFU/well of RV, then on the indicated day pi the cultures were fixed and subjected to the TUNEL assay and immunofluorescence staining. Statistically, a and b are significantly different (one –way anova, $p<0.05$). **A.** The total number of nuclei (DAPI stain) and the number of apoptotic nuclei (TUNEL positive) were calculated using digital analysis for both uninfected and infected CT cultures. The error bars represent the range of results for duplicate wells. The data shown is from one experiment, but the experiment was repeated three times with similar results. **B.** The ratio of apoptotic nuclei (TUNEL positive) to the total nuclei (DAPI stained) as a result of the TUNEL experiments were calculated. The bars represent the average of ratios from three experiments, each experiment used trophoblasts from a different placental preparations. The error bars indicate the standard deviation between experiments.

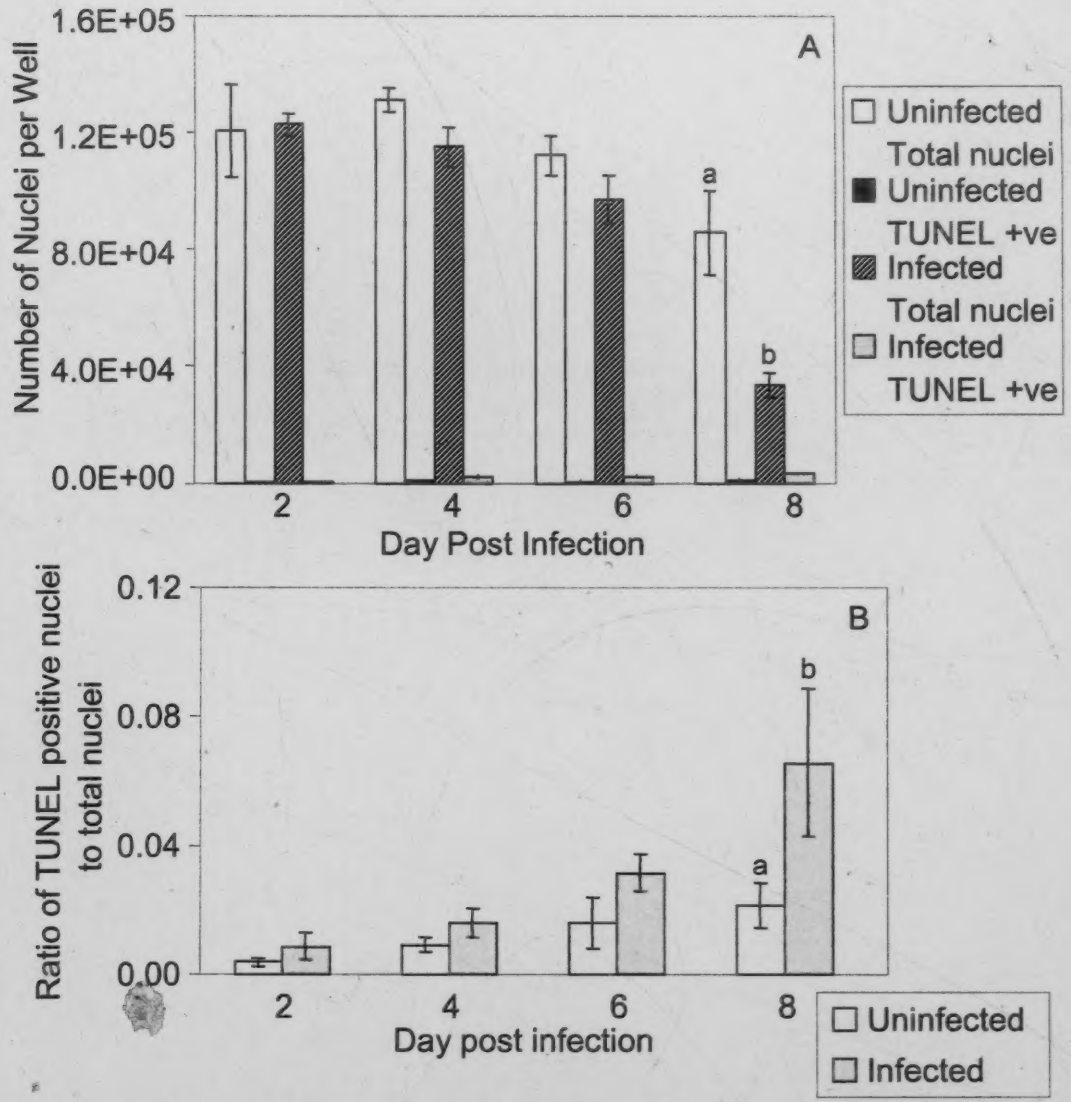


Figure 18. The amount of apoptosis in ST cultures infected with Rubella virus as detected with TUNEL assay. ST were cultured, infected with $1.6x10^6$ PFU/well of RV, then on the indicated day pi the cultures were fixed and subjected to the TUNEL assay and immunofluorescence staining. Statistically, a and b are significantly different (one –way anova, $p<0.05$). **A.** The total number of nuclei (DAPI stain) and the number of apoptotic nuclei (TUNEL positive) were calculated using digital analysis for both uninfected and infected CT cultures. The error bars represent the range of results for duplicate wells. The data shown is from one experiment, but the experiment was repeated three times with similar results. **B.** The ratio of apoptotic nuclei (TUNEL positive) to the total nuclei (DAPI stained) as a result of the TUNEL experiments were calculated. The bars represent the average of ratios from three experiments, each experiment used trophoblasts from a different placental preparations. The error bars indicate the standard deviation between experiments.

The results suggest that the majority of trophoblast loss may not be due to apoptosis during RV infection. The ratio of apoptotic nuclei to the total number of nuclei remaining per well was also examined, and the ratio was higher in the infected cultures than in the controls. The ratios are significantly different for CT on days 4 and 6 pi (Figure 17) and for ST on day 8 pi (Figure 18) and suggest that apoptosis is related to cell loss in the infected cultures. The ratios are the average of three different placental preparations. The standard deviations indicate that the amount of apoptosis compared to the amount of nuclei lost was similar for each different placental preparation. In summary, the TUNEL analysis indicated that the amount of apoptosis in infected trophoblasts was relatively low compared to the amount of cell loss detected, but the ratio of apoptosis to remaining nuclei was higher in the infected cultures.

Since TUNEL alone could not demonstrate whether or not apoptosis was responsible for the cell loss seen, additional methods were carried out to estimate the amount of cell loss due to apoptosis. While an increase in the percent apoptosis is often seen on days when cell loss is high during infection, cell loss also occurs without a large increase in apoptosis. This raises the questions –what is the relationship between apoptosis and infection? Are all the infected cells dying through apoptosis? And are all the apoptotic cells infected?

3.5.1 The Infected Cells in Culture are Non-apoptotic

RV is known to induce apoptosis in some cell lines and my results indicated it induces some apoptosis in trophoblasts (38, 112). The amount of infection in trophoblasts was also low (12% in Figure 8) suggesting that infected cells might be undergoing apoptosis. Therefore, I next examined apoptosis and infection together.

Further, determining what was responsible for the large amount of cell loss was important – was it something other than apoptosis and infection? The digital images of the photomicrographs of TUNEL were overlaid with the photomicrographs of infection and the resulting image was analysed for cells that were dual-fluorescent. The digital analysis determined the majority (82+/-14%) of infected cells (Rhodamine stained RC) were non-apoptotic (as shown in Figure 19, panel h) but that some appeared apoptotic (panel h). Results also indicated that some of the apoptotic cells (FITC-stained nuclei) were not infected. Since TUNEL labels apoptotic nuclei and infection is in the cytoplasm, quantifying the numbers of apoptotic nuclei that are associated with infection is difficult and somewhat subjective. None the less, these results suggested that apoptosis was only one of the mechanisms by which the infected cells were dying. Supporting this hypothesis were experiments showing trophoblast supernatants having a large amount of virus at time points late in infection when very few cells were present (Figure 10). These results suggested that the infected cells were being lysed, releasing the progeny virus into the supernatant, or that cells containing infective virus lose adherence. To summarize the evidence: TUNEL detected a small amount of apoptosis during infection, the amount of nuclei loss was great while the amount of apoptosis was comparably low, and only a portion of the infected cells were apoptotic. Therefore the question arose – what role does apoptosis play in the cell loss? A couple of different methods were used to explore these possibilities – the effect of apoptosis inhibitors on cell loss and examination of the non-adherent cells. These methods allow examination of the role apoptosis plays in cell loss during RV infection and confirmed that the cells are lost through two different mechanisms.

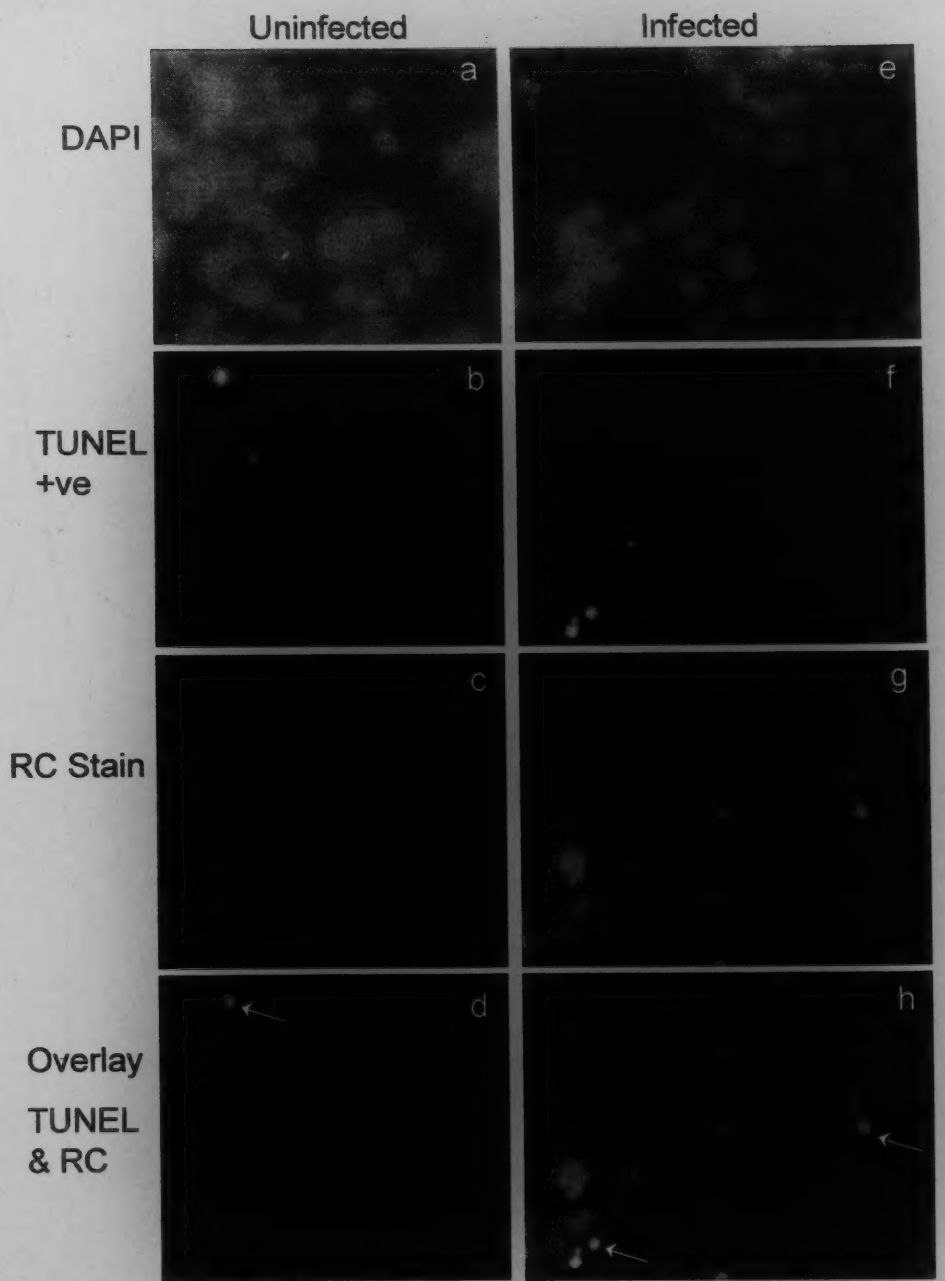


Figure 19. Photomicrographs of ST at day 4 post infection. ST were cultured, infected with $1.6x10^6$ PFU/well of RV, then on day 4 pi the cultures were fixed and subjected to the TUNEL assay and immunofluorescence staining. **a.** DAPI stained total nuclei of uninfected ST. **b.** TUNEL positive nuclei (green) of uninfected ST. **c.** Infection-associated nuclei (RC) of uninfected ST. **d.** Overlay of infection and TUNEL positive nuclei of uninfected ST. White arrow head: apoptosis in absence of infection. **e.** DAPI stained total nuclei of infected ST. **f.** TUNEL positive nuclei of infected ST. **g.** Infection-associated nuclei (RC) (red) of infected ST. **h.** Overlay of infection and TUNEL positive nuclei of infected ST. White arrow head: apoptosis possibly associated with infection. Yellow arrow head: infection without apoptosis.

3.6 Inhibition of Apoptosis during RV Infection

TUNEL analysis indicated that the amount of apoptosis occurring in culture was low and suggested that trophoblasts loss during RV infection was through non-apoptotic mechanisms. Apoptotic inhibitors were used to determine if inhibiting apoptosis during RV infection would block trophoblasts loss. The use of apoptotic inhibitors would confirm whether or not apoptosis was required for the loss of trophoblasts during RV infection. In doing so, it would verify the low amount of apoptosis detected in the TUNEL analysis. Two apoptotic inhibitors were chosen, Z-VAD-FMK and anti-TNFα. Z-VAD-FMK is a broadly acting caspase inhibitor used to inhibit apoptosis induced by multiple stimuli in cells (104, 142). Z-VAD-FMK is relatively non-toxic to cells, can penetrate the cell membrane, and provides irreversible, specific inhibition of all caspase activities. Z-VAD-FMK had previously been used to block RV induced apoptosis in Vero cells (129). An antibody to the death factor TNFα was also utilized because this cytokine induces apoptosis in trophoblasts (165), and is a component in some apoptotic pathways during viral infections (148). As well, the effect of EGF on apoptosis during RV infection was also examined. The growth factor EGF inhibits apoptosis in trophoblasts (53) and the ST are continuously cultured in the presence of EGF, therefore EGF may play a role in inhibiting apoptosis and blocking cell loss.

Z-VAD-FMK was added to the cultures and the amount of apoptosis and cell loss determined as described in methods section 2.6.2. In infected CT cultures at day 4 pi, Z-VAD-FMK was able to lower the amount of apoptosis in both placental preparations, and was able to block some of the cell loss (Figure 20). Z-VAD-FMK inhibited about 75% of the apoptosis during infection in CT but only 35% to 55% of cell loss, depending on the

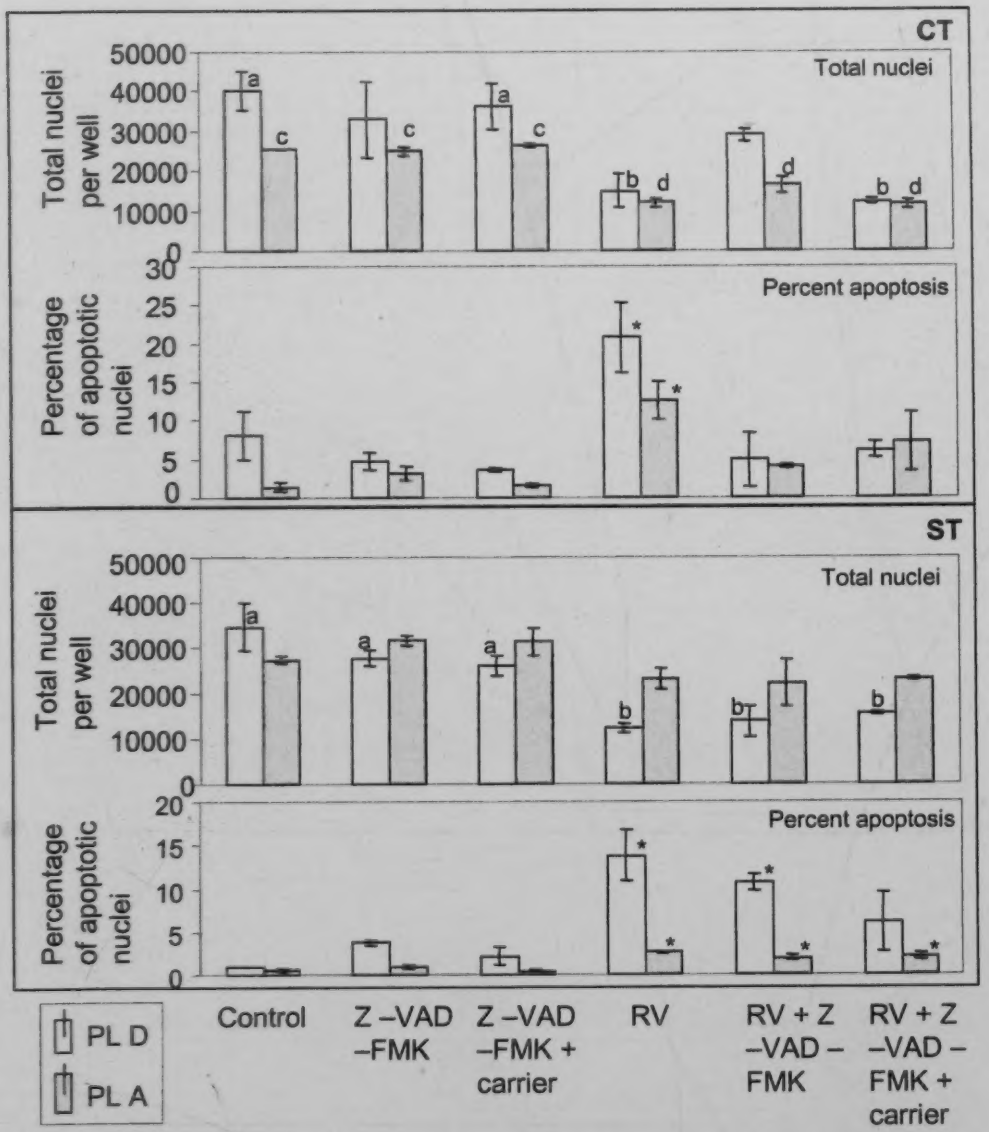


Figure 20. The effect of Z –VAD –FMK on apoptosis and cell loss in Rubella virus infected trophoblasts cultured at 2% FBS. CT (top panel) and ST (lower panel) were cultured in 2% FBS, treated with Z –VAD –FMK, and infected with 1.6×10^6 PFU/well of RV. The CT were fixed on day 4 pi, and the ST were fixed on day 6 pi. Then TUNEL assay, immunofluorescence staining, and digital analysis were carried out. The total number of nuclei and the percentage of apoptosis for two experiments are shown, each with trophoblasts from a different placenta. The bars and symbols represent the average of duplicate wells and the error bars represent the range. Statistically, a and b, or c and d label total nuclei that are significantly different from each other and * labels a percentage of apoptosis that is significantly different from the other unlabelled percentages (one –way anova, $p<0.05$).

placental preparation (Figure 20). In contrast in ST on day 6 pi, Z-VAD-FMK was able to inhibit about 25-30% of the apoptosis, however this was insignificant, and had very little effect on cell loss (Figure 20). It is important to remember that ST are continuously cultured in EGF and this could be having an effect. However, the Z-VAD-FMK carrier, DMSO, independently affected trophoblast apoptosis. The carrier lowered the amount of RV-associated apoptosis in both CT experiments and in one ST experiment but had no effect on cell loss in any experiment. Thus I have little evidence that Z-VAD-FMK was able to block infection-induced apoptosis but some evidence of a minimal effect on cell loss.

Preliminary experiments carried out in 10% FBS showed that anti-TNFα was able to block about 50% of the infection-induced apoptosis in CT. However, repeat experiments cultured in 2% FBS conditions (where cell loss is high) show some effects of anti-TNFα on inhibiting infection-associated apoptosis, although it was not reproducible (Figure 21). Even where anti-TNFα reduced infection-induced apoptosis (CT panel, PL A, Figure 21), the antibody had no effect on blocking the cell loss (Figure 21). These results suggest that TNFα plays a minor role in infection-induced apoptosis and no role in infection-induced cell loss.

To determine whether EGF inhibited apoptosis seen during RV infection of trophoblasts, EGF was added to CT cultures and was removed from ST cultures. In CT, the addition of EGF lowered the amount of apoptosis seen in uninfected cultures, and significantly lowered the amount of apoptosis seen during infection (Figure 22). The addition of EGF was able to inhibit 50-90% of the apoptosis seen in CT during RV infection and blocked some (~25%) of the cell loss, although this was not significant.

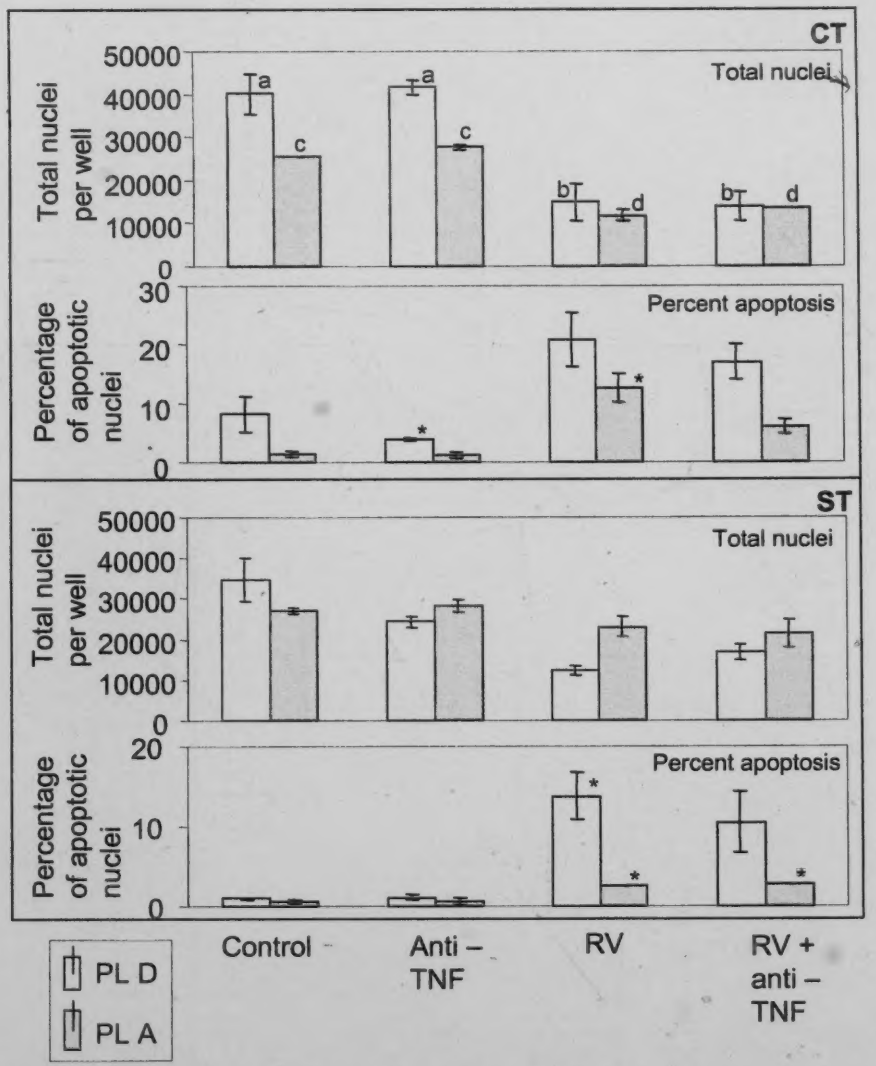


Figure 21. The effect of neutralizing TNFα antibody on apoptosis and cell loss in Rubella virus infected trophoblasts cultured at 2% FBS. CT and ST were cultured in 2% FBS, infected with $1.6x10^6$ PFU/well of RV, and simultaneously treated with anti –TNFα. The CT were fixed on day 4 pi, and the ST were fixed on day 6 pi. Then TUNEL assay, immunofluorescence staining, and digital analysis were carried out. The total number of nuclei and the percentage of apoptosis were calculated for two experiments, each experiment using trophoblasts from a different placenta. The bars and symbols represent the average of duplicate wells and the error bars represent the range. Statistically, a and b, or c and d label total nuclei that are significantly different from each other and * labels a percentage of apoptosis that is significantly different from the other unlabelled percentages (one –way anova, $p<0.05$).

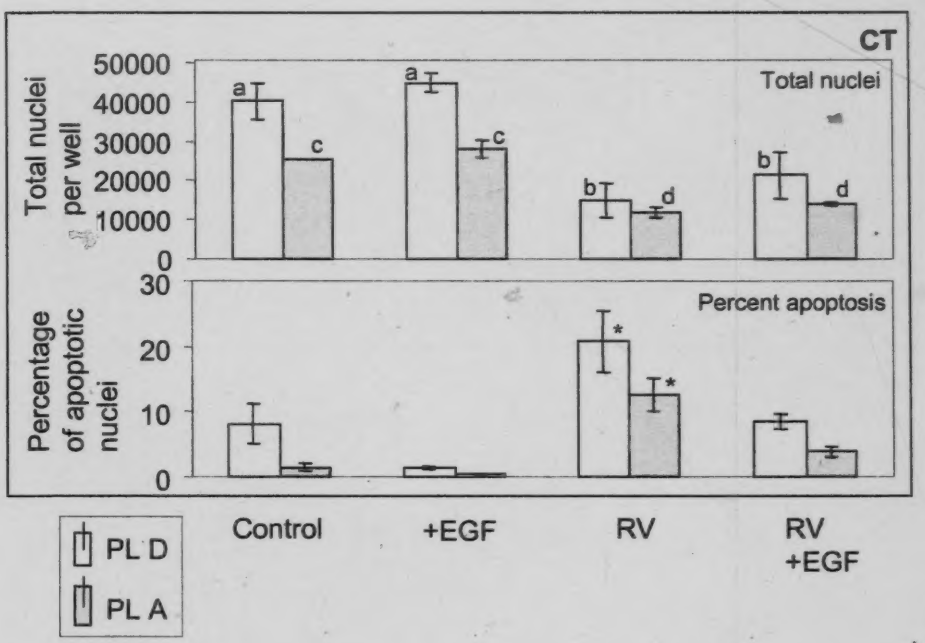


Figure 22. The effect of EGF on apoptosis and cell loss in Rubella virus infected cytotrophoblasts cultured at 2% FBS. The experiments were carried out on CT cultured with and without added EGF, infected with $1.6x10^6$ PFU/well of RV, fixed at day 4 pi, TUNEL assay and immunofluorescence staining carried out, and digital analysis done. The total number of nuclei and the percentage of apoptosis was calculated for two experiments, each with trophoblasts from a different placenta. The bars and symbols represent the average of duplicate wells and the error bars represent the range. Statistically, a and b, or c and d label total nuclei that are significantly different from each other and * labels a percentage of apoptosis that is significantly different from the other unlabelled percentages (one –way anova, $p<0.05$).

Looking at the ST experiment, the removal of EGF increased the amount of apoptosis and allowed for more cell loss in both uninfected and infected cultures, however, the amount depended on the placental preparation (Figure 23). The removal of EGF from ST during infection causes a further increase of 2-4% in the amount of apoptosis detected and an increase of 5000-7000 nuclei in the amount of cell loss measured. Together, Figures 22 and 23 indicate that EGF was able to give the trophoblasts some resistance to apoptosis during RV infection, but had only a small effect on the cell loss seen.

3.7 Infection and Apoptosis in the Non-Adherent Cell Population

Since ≤50% of the cell loss appeared to be due to apoptosis, and only a small portion of the apoptotic cells were RV positive, it was logical to ask if the cells lost were infected, apoptotic or both. Non-adherent cells from RV-infected and uninfected cultures at day 4-9 pi were spun onto glass microscope slides and examined for apoptosis and infection. Figure 24 shows the average number of nuclei recovered and the ratio of nuclei that were apoptotic, RC associated (infected), both, or neither. The number of non-adherent nuclei recovered was higher in the infected cultures than the uninfected cultures (panels C and D), which agreed with the nuclei loss seen in the same experiment. In the uninfected cultures 40+/-8% of the non-adherent nuclei were apoptotic (average of all days pi; data not shown in Figure 24). Figure 24 (panel B, PL E) indicates that the majority of non-adherent nuclei recovered were infected and non-apoptotic (Infected only, ratio of 0.45+/-0.09 on day 6 pi) in the infected cultures. Only a small portion of the cells was apoptotic only, with a ratio of 0.07+/-0.03 for PL E on day 6 pi. Similar to the adherent population, only a portion of the cells was both apoptotic and infected (ratio of 0.14+/-0.06 for PL E on day 6 pi). A rather large portion of the non-adherent cells was

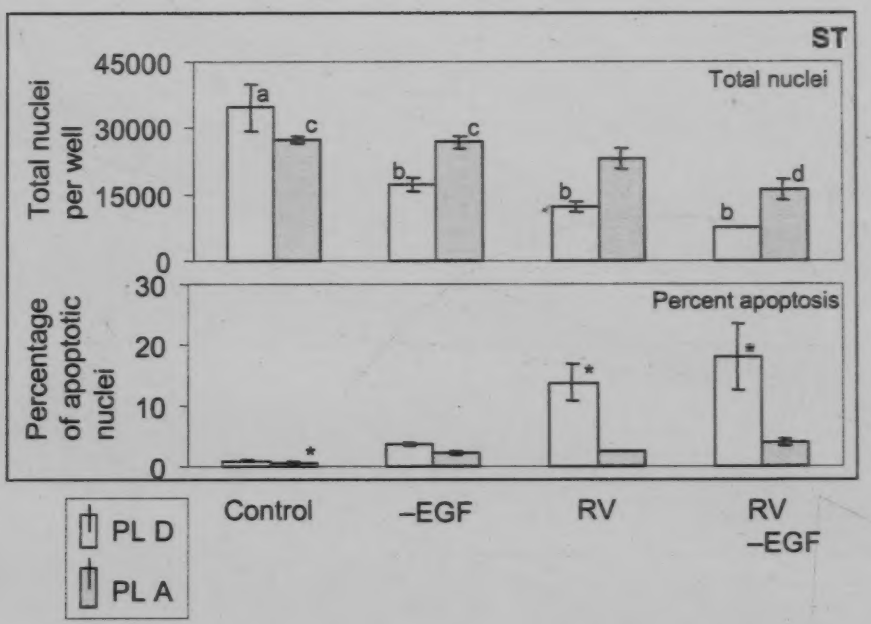


Figure 23. The effect of EGF on apoptosis and cell loss in Rubella virus infected syncytiotrophoblasts cultured at 2% FBS. ST were cultured normally, in the presence of EGF for 5 days. Then some of the cultures had EGF removed simultaneously with RV infection ($1.6x10^6$ PFU/well). The cultures were fixed at day 6 pi, TUNEL assay and immunofluorescence staining carried out, and digital analysis done. The total number of nuclei and the percentage of apoptosis was calculated for two experiments, each with trophoblasts from a different placenta. The bars and symbols represent the average of duplicate wells and the error bars represent the range. Statistically, a and b, or c and d label total nuclei that are significantly different from each other and * labels a percentage of apoptosis that is significantly different from the other unlabelled percentages (one –way anova, $p<0.05$).

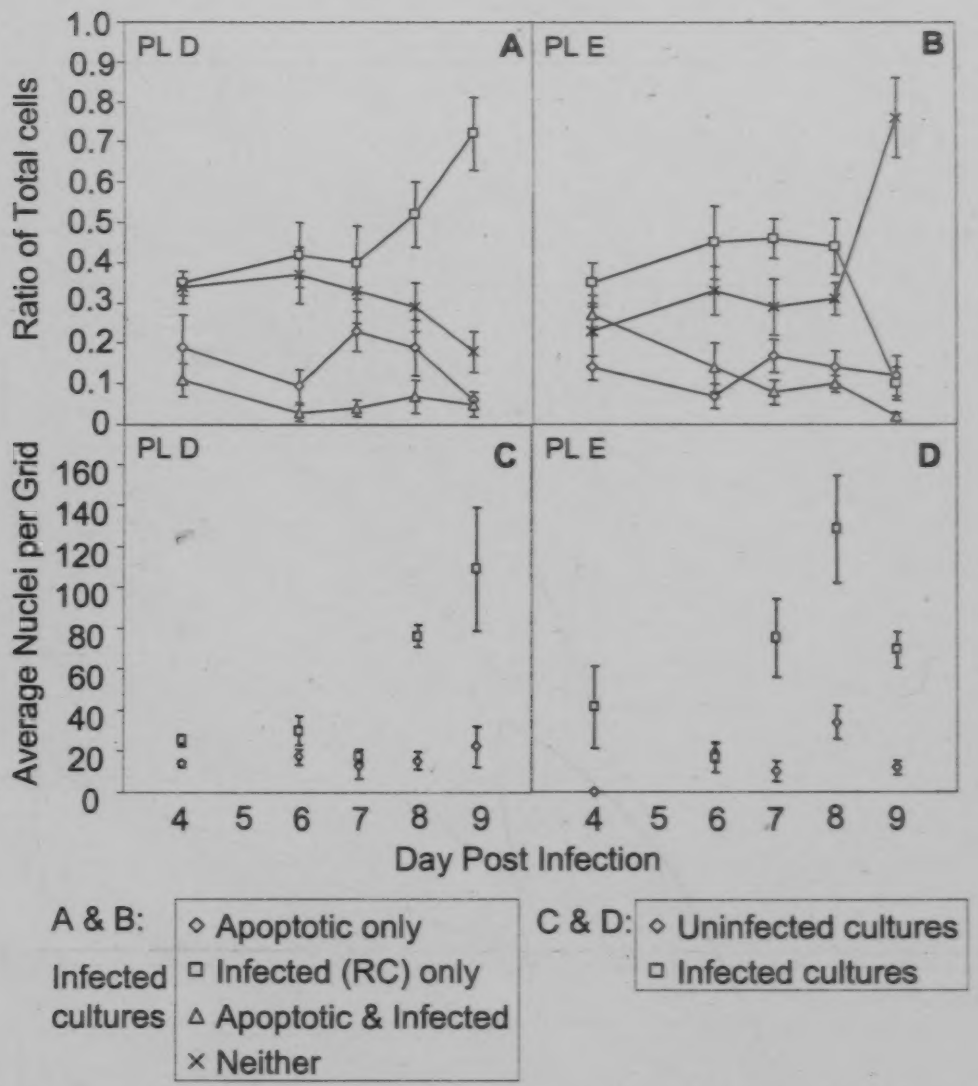


Figure 24. The amount of infection and apoptosis in the non-adherent cell population of ST cultures. The supernatants of ST cultures were collected on the indicated day pi, and the non –adherent cell population spun onto glass microscope slides. The non –adherent cells were fixed and stained for apoptosis and E1 glycoprotein (RC) using the TUNEL assay and immunofluorescence staining, respectively. The average total nuclei counted per slide (**C & D**) were calculated for the uninfected cultures and the infected cultures. The ratio of total cells that were apoptotic, infected, both, or neither (**A & B**) were calculated for the infected culture only. Two experiments are shown, PL D and PL E preparation. The symbols represent the average of five grids counted and the error bars represent the standard deviation.

neither infected nor apoptotic (ratio of 0.33+/-0.06 for PL E on day 6 pi), indicating that some cells were lost due to bystander effects. This experiment revealed that the largest fraction of lost cells were infected, and confirmed that RV infection of trophoblasts induced death through two mechanisms, apoptosis and non-apoptotic cell loss.

CHAPTER 4: DISCUSSION

While it has been known for some time that RV causes CRS, it is unknown exactly how the virus reaches the fetus. The fetus is contained inside several layers of tissue and everything that passes from the maternal blood must travel through the placental barrier, specifically the syncytiotrophoblast, in the villous placenta (8, 96, 138). Previous studies have shown that RV induces damage to the placenta during infection (141). This study proposed that RV was able to reach the fetus by crossing the placental barrier in the villous placenta. I have shown that RV is able to productively infect villous ST, the outermost, and most definitive component, of this placental barrier. I have also shown that even low level ST infection leads to ST disruption. Thus, my studies provide evidence that the outer placental barrier can be infected and disrupted by RV and thereby support my hypothesis that in utero transmission of RV occurs in the villous placenta.

I also hypothesized that RV was able to cross the villous placenta to the fetus through two possible mechanisms –productive infection with virus released basally or destruction of the barrier following infection. When I tested this hypothesis through experimentation, I found that subsequent to RV infection, ST membrane cultures simultaneously lost integrity and infectious virus was found in the basal compartment. These observations imply that RV passes from the mother to the fetus through a damaged villous placenta, but do not disprove the hypothesis of basal release. These results and conclusions are novel; no one has shown RV virus infection of trophoblasts. As well, through this study we have created a good model for investigating the details of trophoblast death and destruction. The study of RV infection in trophoblasts can be used to test new compounds examining the survival and death of villous trophoblasts.

Exploring in detail ways to inhibit trophoblast death under stress, promote trophoblasts survival and innate defence mechanisms.

Rubella is primarily a human disease and even though RV is able to infect a wide variety of cells, its ability to infect and replicate in primary cells is typically lower and slower than in cell lines (47, 129). Since CT and ST are primary cells, the amount of infection was expected to be lower than in the cell line Vero, but the amount of infection actually detected was extremely low even at high doses of virus. RV can also infect polarized cells, however its ability to infect polarized cell lines through either the apical or basal surface depends on the cell line or primary cell type (51). I have shown that the infection of polarized ST is possible through the apical surface, which is appropriate for maternal hematogenous infections. However infection wasn't tested through the basal surface. Since, CT had a higher amount of infection detected than ST, and given that ST were cultured in the presence of EGF, which stimulates differentiation in trophoblasts, the maturation state of trophoblasts may play a role in the maximum amount of infection possible. As well, ST are unable to replicate (mitotically inactive) and have limited amounts of transcription ongoing in the cell (12, 75), which may have an effect on the ability of RV to infect and replicate in the outer placental barrier. Indeed, mature ST are very resistant to infection by adenovirus (105), HSV (87), and CMV (61), possibly because of a deficiency in virus receptors (88).

During virus production, the majority of RV can be either released into the supernatant or remained cell associated, depending on the cell type (11). The pattern of production of RV in CT and ST was similar to that seen in Vero, in that the majority of the progeny virus was released into the supernatant. As well, the ability of RV to be

propagated and secreted from polarized cells varies depending on the cell line (51). Some cells are able to secrete virus from both surfaces while some prefer either the apical or basal surface. Because the rapid onset of cell loss in infected cultures can be due to cytolysis, we have no definitive evidence that RV is at all secreted from these cells. Further, the rapid loss of integrity in ST membrane cultures precludes conclusions of polarized release.

The effect of RV infection in cells varies: CPE is not always lethal, and RV can develop persistent infections (48). In CT and ST, detrimental effects were seen during RV infection: CPE was induced, and large numbers of cells were lost. This cell loss was determined to be through primarily non-apoptotic mechanisms, but some apoptosis was involved. Rubella is known to induce apoptosis during infection in several cells –Vero, RK13, and BHK21 –and the level of apoptosis varies with each cell type (38, 129). During RV infection, apoptosis can be induced by the capsid protein, and can involve both p53 and caspases (38, 39, 112). The cytopathic effect of RV on Vero cells is due to apoptosis and can be inhibited by Z-VAD-FMK, indicating the involvement of caspases (129). Trophoblasts are able to undergo apoptosis and actually use apoptosis as a self-regulating mechanism to destroy aging cells (75, 90, 144). In this study, RV infection of trophoblasts also involved some apoptosis that could be inhibited by Z-VAD-FMK. In the trophoblast cultures it is unknown what induces apoptosis during infection, or if it can be induced by the RV capsid protein. TNFα is a potent inducer of apoptosis in trophoblasts (53, 165) and TNFα production can be stimulated with viral infection in trophoblasts and other cell types (20, 91); however, TNFα does not play a role in the induction of apoptosis during RV infection of trophoblasts. The TUNEL assay, which

stains nuclei undergoing apoptosis, indicated that very few nuclei and a relatively low percentage of the adherent cells were actually apoptotic in RV-infected cultures. My experiments with the non-adherent compartment of RV-infected ST cultures shows many lost cells are infected but not apoptotic, and indicates that apoptosis is only one of the mechanisms responsible for trophoblast loss during RV infection. Indeed, it appears that the majority of infected cells are dying due to non-apoptotic cell loss and suggests that apoptosis plays a secondary role in the RV infection of trophoblasts. This is similar to findings by Martinez and Zapata (2002) who state that most of the apoptotic cells where non-infected but were nearby infected cells (109). The factors responsible for the induction of this non-apoptotic cell loss are unknown, and it remains to be determined whether RV proteins play a direct role. There is no evidence to date to suggest that RV is protecting these non-apoptotic, infected cells from undergoing apoptosis.

While non-apoptotic cell loss is proposed as the primary mechanism for death, it may be possible that trophoblasts are lost from culture due to a loss of adherence during RV infection. The two largest non-adherent cell populations were non-apoptotic infected and uninfected cells. Examining the non-adherent cell population did not confirm that all the lost nuclei were dead. The mechanism of cell loss could include a loss of adherence affecting both infected and surrounding uninfected trophoblasts. Epithelial cells that lose adherence are known to eventually enter a specialized form of apoptosis termed anoikis (49).

There are several viruses and pathogens that can infect the fetal tissue as a result of maternal infection and consequently cause damage to the growing fetus. CMV has also been shown to infect trophoblasts in vitro (43, 62). The way CMV infection acts on

trophoblasts is different from the way RV acts. CMV infection in trophoblasts leads to cell loss due to TNFα-induced apoptosis in the first 24 hours (20). This is unlike RV infection where the trophoblast death is due to both apoptosis and non-apoptotic cell loss neither of which appears TNF dependant. Interestingly, trophoblast apoptosis and cell loss is also induced by infection with the protozoan parasite Toxoplasma gondii (1), which also causes congenital abnormalities (77). However, most apoptotic trophoblasts in T. gondii-infected cultures are uninfected. On the other hand, like RV, T. gondii infection-induced trophoblast cell loss and apoptosis is TNF-independent. So while there are various infectious pathogens that cause congenital abnormalities, each may act differently upon the villous placenta in reaching the fetal tissue.

As with any project, difficulties arise and we must work through them to obtain results and information regarding our hypothesis. The large amount of death proved to be a general difficulty, especially in the case of the basal release experiments, although this alone provided a key point of information regarding the infection of trophoblasts with RV. Other issues of concern included the low amount of infection detected and the low amount of apoptosis detected. Why was there so much death with so little infection and apoptosis? Determining the amount of infection seen in the trophoblasts cultures was relatively easy, however the amounts were low compared to the amounts detected in Vero cells. However, examining the non-adherent population indicated that at least half of the dying cells were infected and non-apoptotic, suggesting that there was more infection than was detected at any point in time. As well, the high amount of virus production in ST on day 8 (Figure 10) coincides with a high amount of ST loss on day 8 (Figure 16). This suggests that infected cells do not remain on the plate in culture for very long,

indicating the possibility that infected cells lose adherence and are lost from culture before they die. The fact that apoptosis is low both in the cells in culture and the non-adherent population suggests that apoptosis plays a minor role in the death of trophoblasts during infection.

I have shown that RV is able to infect villous trophoblasts, in vitro. While the amount of infection was low compared to commonly used cell lines, the cytopathic effect of this infection was substantial. Based on results presented in this thesis, I propose a mechanism for RV passage across the villous placenta. As depicted in Figure 25, I propose that RV attaches to the apical surface of the ST layer and infects it (Figure 25A-1). Once inside the ST, RV is able to undergo replication. A productive infection develops with the progeny virus released into the surrounding extracellular space (Figure 25A-2) accompanied by ST disruption through both apoptotic and non-apoptotic cell loss (Figure 25B-3). Importantly, my results suggest that the underlying CT can either be infected or concomitantly lost with regional ST disruption. This loss of CT is detrimental to the protection of the fetus in that CT are responsible for the replenishment of the ST layer. Some ST nuclei undergo apoptosis during RV infection (Figure 25B-4). With the loss of CT and ST, the placental barrier becomes severely compromised, and RV can then pass through the holes in the damaged layer and infect the fetal tissue in the villous stroma (Figure 25B-5). Loss of the trophoblast barrier is accompanied by villous inflammation (villitis), which can further damage remaining physical barriers: the trophoblast basement membrane (TBM) and villous vascular endothelium. RV is able to infect fetal tissue, as indicated through pathology studies (37, 141, 150), and, depending

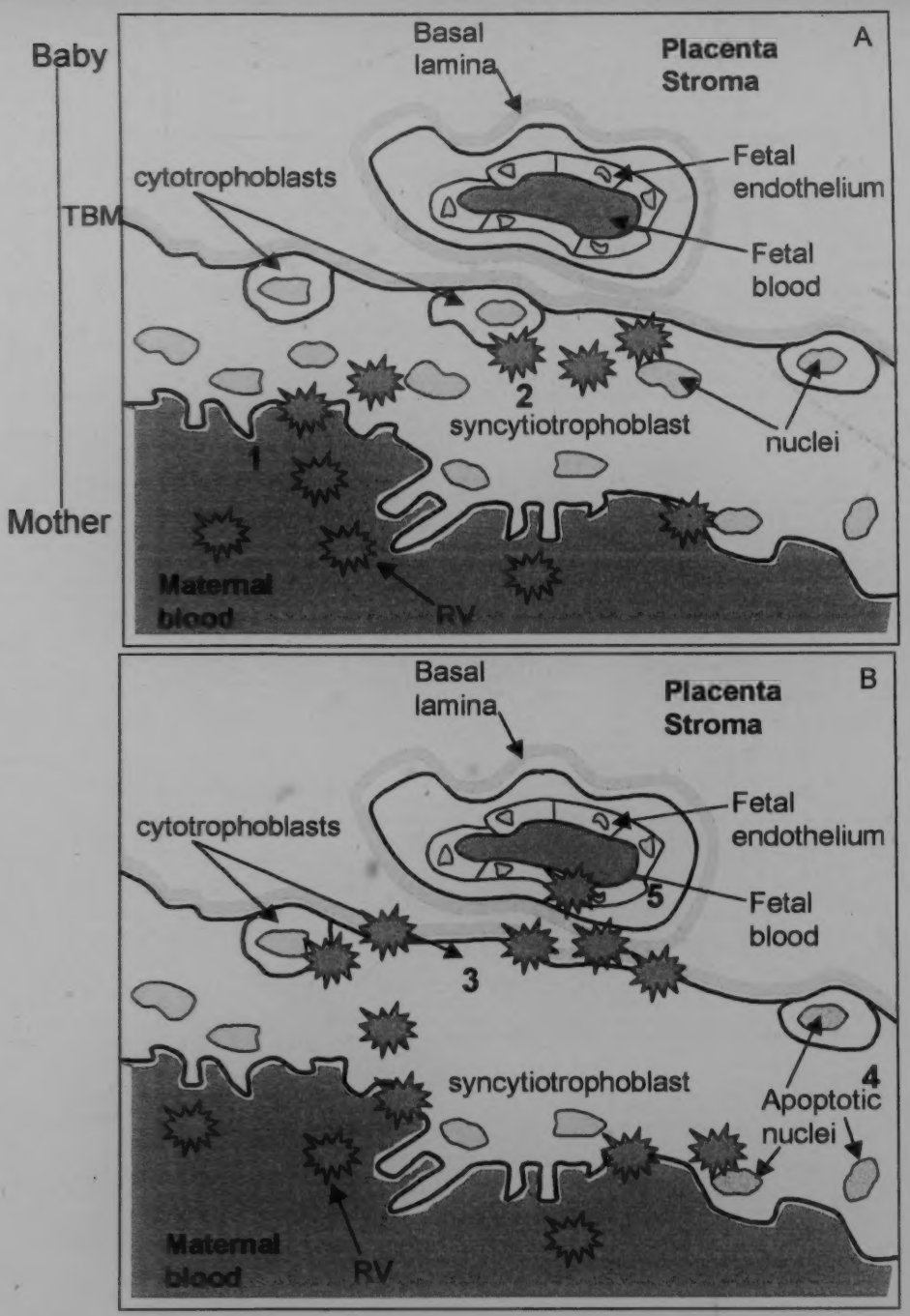


Figure 25. Model of virus passing through the placental barrier. A. Rubella virus (RV; blue) in the maternal blood infects the ST. As infection progresses the CT become infected, apoptosis begins (green nuclei), and cell loss occurs. **B.** The infection resulted in lost nuclei, and the virus is able to pass through the barrier and reach the fetal tissue. The apoptotic nuclei are not always associated with infection. (TBM: trophoblast basement membrane)

on the stage of pregnancy, CRS may develop. Thus, once ST infection begins, reaching the fetal tissue would seem inevitable.

As there are many unanswered questions in this study, there is more work to be done looking at the mechanisms of RV infection of the trophoblast layer and infection of the fetal tissue. One aspect is to determine which factors, if any, are produced and released by the trophoblasts during infection that could induce apoptosis, non-apoptotic cell loss, or cell release. One approach would be to determine whether the transfer of supernatants from the infected trophoblasts to uninfected trophoblasts could also transfer death. This in conjunction with Z-VAD-FMK treatment may be able to tell us if the apoptotic death is due to a soluble, released factor. Given the previous literature (39), looking at whether the capsid protein of RV alone can induce apoptosis in trophoblast is also a good approach. Determining whether virus release from the cell induces non-apoptotic cell loss or whether some released factor is responsible is also important. As part of investigating the death of trophoblasts during RV infection, it is important to consider the idea that infection may be causing the cells to lose adherence and not necessary inducing immediate death. Determining whether RV or trophoblasts are able to cause a loss of adherence during infection is important. A second point of interest is whether trophoblasts may be producing factors that can limit the amount of RV infection. It is interesting to note that trophoblasts are rather resistant to infection but very sensitive to RV-induced disruption. Thus, are interferons or some other innate defense factor being released to inhibit infection and do these participate in trophoblast demise? This could provide some information on the ability of trophoblasts to be infected with RV in vivo, and the ability of other viruses to infect trophoblasts. Following up on the basal release

experiments does not seem to be important for RV infection, since RV induces large amounts of cell damage which would seem sufficient enough to reach the fetal tissue. However, more detailed studies at early times pi may reveal the polarity of release.

The topic of placental infection is still a real area for concern, as the risk of placental infection is still possible even today. While RV has decreased dramatically since the introduction of a vaccine in the late 1960's CRS still occurs, even in North America. Examining RV infection in purified human placental trophoblasts has some relevance, both to how RV induces damage and how other viruses may induce damage, both placental and fetal damage.

APPENDIX A

Placenta Preparations

Assigned Name	Record name
PL A	November 9, 2000
PL B	November 17, 2000
PL C	March 20, 2001
PL D	October 15, 2001
PL E	August 8, 2002

BIBLIOGRAPHY

1. **Abbasi, M., K. Kowalewska-Growchowska, M. A. Bahar, R. T. Kilani, B. Winkler-Lowen, and L. J. Guilbert.** in press. Infection of placental trophoblasts by toxoplasma gondii. J. Infect. Dis.
2. **Aboagye-Mathiesen, G., F. D. Toth, H. Hager, M. Zdravkovic, P. M. Petersen, J. A. Villadsen, V. Zachar, and P. Ebbesen.** 1993. Human trophoblast interferons. Antiviral Research **22:**91-105.
3. **Aboagye-Mathiesen, G., F. D. Toth, M. Zdravkovic, and P. Ebbesen.** 1996. Functional Characteristics of Human Trophoblast Interferons. American Journal of Reproductive Immunology **35:**309-317.
4. **Aboudy, Y., A. Fogel, B. Barnea, E. Mendelson, L. Yosef, T. Frank, and E. Shalev.** 1997. Subclinical Rubella Reinfection during Pregnancy Followed by Transmission of Virus to the Fetus. Journal of Infection **34:**273-76.
5. **Adamo, M. P., M. Leon Monzon, C. Cuffini, M. Pedranti, and M. Zapata.** 2002. Detection of Rubella virus induced apoptosis in vero cell cultures with hematoxylin and eosin staining. Rev Argent Microbiol **34:**177-85.
6. **Aksit, S., A. Egemen, T. Ozacar, Z. Kurugol, P. Keskinoglu, M. Tasbakan, and S. Caglayan.** 1999. Rubella seroprevalence in an unvaccinated population in Izmir: recommendations for rubella vaccination in Turkey. The Pediatric Infectious Disease Journal **18:**577-80.
7. **Altshuler, G., and P. Russell.** The Human Placental Villitides: A Review of Chronic Intrauterine Infection.63-112.
8. **Aplin, J. D.** 1991. Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence *in vivo* and *in vitro*. Journal of Cell Science **99:**681-92.
9. **Arechavaleta-Velasco, F., H. Koi, J. F. r. Strauss, and S. Parry.** 2002. Viral infection of the trophoblast: time to take a serious look at its role in abnormal implantation and placentation? J Reprod Immunol. **55:**113-21.
10. **Ashley, C. A.** 1965. Study of the Human Placenta with the Electron Microscope. Archives of Pathology **80:**377-390.
11. **Bardeletti, G., J. Tekoff, and D. Gautheron.** 1979. Rubella virus maturation and production in two host cell systems. Intervirology **11:**97-103.
12. **Benirschke, K., and P. Kaufmann.** 1990. Pathology of the Human Placenta, 2nd ed. Springer-Verlag New York Inc., New York.
13. **Boise, L. H., M. Gonzalez-Garcia, C. E. Postema, L. Ding, T. Lindsten, L. A. Turka, X. Mao, G. Nunez, and C. B. Thompson.** 1993. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell **74:**597-608.
14. **Branch, D. R., A. Shah, and L. J. Guilbert.** 1991. A specific and reliable bioassay for the detection of femtomolar levels of human and murine tumor necrosis factors. Journal of Immunological Methods **143:**251-261.
15. **Brent, R. L.** 1986. Editorial Comment: Definition of a Teratogen and the Relationship of Teratogenicity to Carcinogenicity. Teratology **34:**359-60.

16. **Bullens, D., K. Smets, and P. Vanhaesebrouck.** 2000. Congenital rubella syndrome after maternal reinfection. Clin Pediatr (Phila). **39:**113-6.
17. **Burton, G. J., and A. L. Watson.** 1997. The Structure of the Human Placenta: Implications for Initiating and Defending Against Virus Infections. Reviews in Medical Virology **7:**219-228.
18. **Cande, C., F. Cecconi, P. Dessen, and G. Kroemer.** 2002. Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? J Cell Sci. **115:**4727-34.
19. **Cande, C., I. Cohen, E. Daugas, L. Ravagnan, N. Larochette, N. Zamzami, and G. Kroemer.** 2002. Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie. **84:**215-22.
20. **Chan, G., D. G. Hemmings, A. D. Yurochko, and L. J. Guilbert.** 2002. Human cytomegalovirus-caused damage to placental trophoblasts mediated by immediate-early gene-induced tumor necrosis factor-alpha. American Journal of Pathology **161:**1371-81.
21. **Chantler, J. K., and A. J. Tingle.** 1980. Replication and Expression of Rubella Virus in Human Lymphocyte Populations. Journal of General Virology **50:**317-28.
22. **Chen, J. P., J. H. Strauss, E. G. Strauss, and T. K. Frey.** 1996. Characterization of the rubella virus nonstructural protease domain and its cleavage site. J Virol **70:**4707-13.
23. **Chernesky, M. A., and J. B. Mahony.** 1995. Rubella Virus, p. 968-973. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of CLinical Microbiology, 6th ed. American Society for Microbiology, Washington.
24. **Chieux, V., W. Chehadeh, J. Harvey, O. Haller, P. Wattre, and D. Hober.** 2001. Inhibition of Coxsackievirus B4 Replication in Stably Transfected Cells Expressing Human MxA Protein. Virology **283:**84-92.
25. **Chittenden, T., H. E.A., R. O'Connor, C. Flemington, R. J. Lutz, G. I. Evan, and B. C. Guild.** 1995. Induction of apoptosis by the Bcl-2 homologue Bak. Nature **374:**733-6.
26. **Conrad, K. P., and D. F. Benyo.** 1997. Placental Cytokines and the Pathogenesis of Preeclampsia. American Journal of Reproductive Immunology **37:**240-249.
27. **Creagh, E. M., and S. J. Martin.** 2001. Caspases: cellular demolition experts. Biochemical Society Transactions **29:**696-702.
28. **Cusi, M. G., P. E. Valensin, and C. Cellesi.** 1993. Possibility of reinfection after immunisation with RA27/3 live sttenuated rubella virus. Archives of Virology **129:**337-40.
29. **Cutts, F. T., S. E. Robertson, J. L. Diaz-Ortega, and R. Samuel.** 1997. Control of rubella and congenital rubella syndrome (CRS) in developing countries, part 1: burden of disease from CRS. Bull World Health Organ. **75:**55-68.
30. **Cutts, F. T., and E. Vynnycky.** 1999. Modelling the incidence of congenital rubella syndrome indeveloping countries. International Journal of Epidemiology **28:**1176-1184.
31. **Dash, P.** 2003, posting date. Apoptosis - Caspases. [Online.]
32. **Dash, P.** 2003, posting date. Apoptosis - Death Receptors. [Online.]

33. **Dash, P.** 2003, posting date. Apoptosis - Mitochondria. [Online.]
34. **Diaz, M. O., S. Ziemin, M. M. Le Beau, P. Pitha, S. D. Smith, R. R. Chilcote, and J. D. Rowley.** 1988. Homozygous deletion of the α- and β_1-interferon genes in human leukemia and derived cell lines. Proceedings of the National Academy of Science, USA **85:**5259-63.
35. **Dickinson, J. E.** 1994. Viral Teratology, p. 12-17. *In* B. Gonik (ed.), Viral Diseases in Pregnancy. Springer-Verlag New York, Inc., New York.
36. **Domegan, L. M., and G. J. Atkins.** 2002. Apoptosis induction by the Terien and vaccine RA27/3 strains of rubella virus causes depletion of oligodendrocytes from rat neural cell cultures. J Gen Virol **83:**2135-43.
37. **Driscoll, S. G.** 1969. Histopathology of Gestational Rubella. American Journal of Diseases in Children **118:**49-53.
38. **Duncan, R., J. Muller, N. Lee, A. Esmaili, and H. L. Nakhasi.** 1999. Rubella Virus-Induced Apoptosis Varies among Cell Lines and Is Modulated by Bcl-X_L and Caspase Inhibitors. Virology **255:**117-128.
39. **Duncan, R. C., A. Esmaili, L. M. Law, S. Bertholet, C. Hough, T. C. Hobman, and H. L. Nakhasi.** 2000. Rubella virus capsid protein induces apoptosis in transfected RK13 cells. Virology **275:**20-29.
40. **Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata.** 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature **391:**43-50.
41. **Enders, A.** 1965. A Comparative Study of the Fine Structure of the Trophoblast in Several Hemochorial Placentas. American Journal of Anatomy **116:**29-68.
42. **Farrow, S. N., J. H. White, I. Martinou, T. Raven, K. T. Pun, C. J. Grinham, J. C. Martinou, and R. Brown.** 1995. Cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K. Nature **374:**731-3.
43. **Fisher, S., O. Genbacev, E. Maidji, and L. Pereira.** 2000. Human Cytomegalovirus Infection of Placental Cytotrophoblasts In Vitro and In Utero: Implications for Transmission and Pathogenesis. Journal of Virology **74:**6808-6820.
44. **Forng, R. Y., and T. K. Frey.** 1995. Identification of the rubella virus nonstructural proteins. Virology **206:**843-53.
45. **Fox, H.** 1997. Pathology of the Placenta, 2nd ed, vol. 7. W. B. Saunders Company Limited, London.
46. **Freathy, C., D. G. Brown, R. A. Roberts, and K. Cain.** 2000. Transforming growth factor-beta(1) induces apoptosis in rat FaO hepatoma cells via cytochrome c release and oligomerization of Apaf-1 to form a approximately 700-kd apoptosome caspase-processing complex. Hepatology. **32:**750-60.
47. **Frey, T. K.** 1994. Molecular biology of rubella virus. Adv Virus Res **44:**69-160.
48. **Frey, T. K., and J. S. Wolinsky.** 1999. Rubella Virus (Togaviridae), p. 1592-1601. *In* A. Granoff and R. G. Webster (ed.), Encyclopedia of VIrology, 2nd ed, vol. 3. Academic Press, San Diego.
49. **Frisch, S. M., and H. Francis.** 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol **124:**619-26.
50. **Fujino, T., I. Iwamoto, H. Otsuka, T. Ikeda, S. Takesako, and Y. Nagata.** 1999. Apoptosis in Placentas From Human T-Lymphotropic Virus Type I-

Seropositive Pregnant Women: A Possible Defense Mechanism Against Transmission From Mother to Fetus. Obstetrics and Gynecology **94:**279-283.

51. **Garbutt, M., H. Chan, and T. C. Hobman.** 1999. Secretion of Rubella Virions and Virus-like Particles in Cultured Epithelial Cells. Virology **261:**340-6.
52. **Garcia, A. G. P., R. L. S. Marques, Y. Y. Lobato, M. E. F. Fonseca, and M. D. Wigg.** 1985. Placental Pathology in Congenital Rubella. Placenta **6:**281-95.
53. **Garcia-Lloret, M. I., J. Yui, B. Winkler-Lowen, and L. J. Guilbert.** 1996. Epidermal Growth Factor Inhibits Cytokine-Induced Apoptosis of Primary Human Trophoblasts. Journal of Cellular Physiology **167:**324-32.
54. **Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson.** 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. The Journal of Cell Biology **119:**493-501.
55. **Gibson, L., S. P. Holmgreen, D. C. Huang, O. Bernard, N. G. Copeland, N. A. Jenkins, G. R. Sutherland, E. Baker, J. M. Adams, and S. Cory.** 1996. bcl-w, a novel member of the bcl-2 family, promotes cell survival. Oncogene **13:**665-75.
56. **Gil, J., and M. Esteban.** 2000. Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): Mechanism of action. Apoptosis **5:**107-114.
57. **Gray, J. E.** 1960. Rubella in Pregnancy. British Medical Journal:1388-90.
58. **Gyorkos.** 1998. JAMC **159:**1091-97.
59. **Health-Canada** 1997, posting date. Outbreak of Rubella Originating Among High-School Students - Selkirk, Manitoba. Health Protection Branch - Laboratory Centre for Disease Control Volume 23-13. [Online.]
60. **Health-Canada** 1996, posting date. Survey of Congenital Rubella Syndrome, Montreal, Laval, and Monteregie, Quebec, 1985-1991. Health Protection Branch - Laboratory Centre for Disease Control Volume 22-5. [Online.]
61. **Hemmings, D. G., G. Chan, and L. J. Guilbert.** MScript in preparation.
62. **Hemmings, D. G., R. Kilani, C. Nykiforuk, J. Preiksaitis, and L. J. Guilbert.** 1998. Permissive Cytomegalovirus Infection of Primary Villous Term and First Trimester Trophoblasts. J Virol **72:**4970-79.
63. **Hemmings, D. G., B. Lowen, R. Sherburne, G. Sawicki, and L. J. Guilbert.** 2001. Villous Trophoblasts Cultured on Semi-permeable Membranes form an Effective Barrier to the Passage of High and Low Molecular Weight Particles. Placenta **22:**70-79.
64. **Hemphill, M. L., R. Y. Forng, E. S. Abernathy, and T. K. Frey.** 1988. Time course of virus-specific macromolecular synthesis during rubella virus infection in Vero cells. Virology **162:**65-75.
65. **Hobbins, T. E., and K. O. Smith.** 1968. Fluorescent Plaque Assay of Rubella Virus Infectivity. P. S. E. B. M. **129:**407-412.
66. **Hobman, T. C., and S. Gillam.** 1989. In Vitro and in Vivo Expression of Rubella Virus Glycoprotein E2: The Signal peptide Is Contained in the C-Terminal Region of Capsid Protein. Virology **173:**241-250.
67. **Hobman, T. C., H. F. Lemon, and K. Jewell.** 1997. Characterization of an Endoplasmic Reticulum Retention Signal in the Rubella Virus E1 Glycoprotein. Journal of Virology **71:**7670-7680.

68. **Hobman, T. C., M. L. Lundstrom, and S. Gillam.** 1990. Processing and Intracellular Transport of Rubella Virus Structural Proteins in COS Cells. Virology **178:**122-133.
69. **Hobman, T. C., M. L. Lundstrom, C. A. Mauracher, L. Woodward, S. Gillam, and M. G. Farquhar.** 1994. Assembly of Rubella Virus Structural Proteins into Virus-like Particles in Transfected Cells. Virology **202:**574-585.
70. **Hobman, T. C., R. Shukin, and S. Gillam.** 1988. Translocation of Rubella Vrius Glycoprotein E1 into the Endoplasmic Reticulum. Journal of Virology **62:**4259-4264.
71. **Hobman, T. C., L. Woodward, and M. G. Farquhar.** 1992. The Rubella Virus E1 Glycoprotein Is Arrested in a Novel Post-ER, Pre-Golgi COmpartment. The Journal of Cell Biology **118:**795-811.
72. **Hobman, T. C., L. Woodward, and M. G. Farquhar.** 1993. The Rubella Virus E2 and E1 Spike Glycoproteins Are Targeted to the Golgi Complex. The Journal of Cell Biology **121:**269-281.
73. **Hofmann, J., M. W. R. Pletz, and U. G. Liebert.** 1999. Rubella virus-induced cytopathic effect in vitro is caused by apoptosis. Journal of General Virology **80:**1657-64.
74. **Huppertz, B., H.-G. Frank, J. C. P. Kingdom, F. Reister, and P. Kaufmann.** 1998. Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. Histochem Cell Biol **110:**495-508.
75. **Huppertz, B., H.-G. Frank, F. Reister, J. Kingdom, H. Korr, and P. Kaufmann.** 1999. Apoptosis Cascade Progresses during Turnover of Human Trophoblast: ANalysis of Villous Cytotrophoblast and SYncytial Fragments In Vitro. Laboratory Investigation **79:**1687-1702.
76. **Jones, C. J. P., and H. Fox.** 1991. Ultrastructure of the Normal Human Placenta. Electron Microsc. Reviews **4:**129-78.
77. **Jones, J. L., A. López, M. Wilson, J. Schulkin, and R. Gibbs.** 2001. Congenital toxoplasmosis: a review. Obstetrics and Gynecology Survey **56:**296-305.
78. **Joshi, V. V.** 1994. Handbook of Placental Pathology. Igaku-Shoin, New York.
79. **Karchmer, A. W., and K. L. Herrmann.** 1969. Comparative Studies of Rubella Vaccines. American Journal of Diseases in Children **118:**197-202.
80. **Katow, S., and A. Sugiura.** 1988. Low pH-induced conformational change of rubella virus envelope proteins. J Gen Virol **69:**2797-2807.
81. **Keith, C. G.** 1991. Congenital rubella infection from reinfection of previously immunised mothers. Aust N Z J Ophthalmol **19:**257.
82. **Khan, N. A., and S. N. Kazzi.** 2000. Yield and costs of screening growth-retarded infants for torch infections. Am J Perinatol **17:**131-35.
83. **Kilani, R. T., L.-J. Chang, M. I. Garcia-Lloret, D. Hemmings, B. Winkler-Lowen, and L. J. Guilbert.** 1997. Placental Trophoblasts Resist Infection by Multiple Human Immunodeficiency Virus (HIV) Type 1 Variants Even with Cytomegalovirus Coinfection but Support HIV Replication after Provirus Transfection. J Virol **71:**6359-6372.
84. **Kimball, J. W.** February 6 2003, posting date. Apoptosis. [Online.]

85. **Kimberlin, D. W.** 1997. Rubella Virus, p. 1257-72. *In* D. D. Richman, R. J. Whitley, and F. G. Hayden (ed.), Clinical Virology. Churchill Livingstone Inc., New York.

86. **Kliman, H. J., J. E. Nestler, E. Sermasi, J. M. Sanger, and J. F. Strauss III.** 1986. Purification, Characterization, and *in Vitro* Differentiation of Cytotrophoblasts from Human Term Placentae. Endocrinology **118:**1567-82.

87. **Koi, H., J. Zhang, A. Makrigiannakis, S. Getsios, C. D. MacCalman, J. F. Strauss III, and S. Parry.** 2002. Syncytiotrophoblast Is a Barrier to Maternal-Fetal Transmission of Herpes Simplex Virus. Biology of Reproduction **67:**1572-1579.

88. **Koi, H., J. Zhang, and S. Perry.** 2001. The Mechanisms of Placental Viral Infection. Annuals of the New York Academy of Science **943:**148-56.

89. **Law, L. M. J., J. C. Everitt, M. D. Beatch, C. F. B. Holmes, and T. C. Hobman.** 2003. Phosphorylation of Rubella Virus Capsid Regulates Its RNA Binding Activity and Virus Replication. J Virol **77:**in press.

90. **Lea, R. G., S. C. Riley, C. Antipatis, L. Hannah, C. J. Ashworth, D. A. Clark, and H. O. D. Critchley.** 1999. Cytokines and the Regulation of Apoptosis in Reproductive Tissues: A Review. American Journal of Reproductive Immunology **42:**100-109.

91. **Lee, B. N., N. Ordonez, E. J. Popek, J. G. Lu, A. Helfgott, N. Eriksen, H. Hammill, C. Kozinetz, M. Doyle, M. Kline, C. Langston, W. T. Shearer, and J. M. Reuben.** 1997. Inflammatory cytokine expression is correlated with the level of human immunodeficiency virus (HIV) transcripts in HIV-infected placental trophoblastic cells. J Virol **71:**3628-35.

92. **Lee, J. Y., and D. S. Bowden.** 2000. Rubella Virus Replication and Links to Teratogenicity. Clinical Microbiology Reviews **13:**571-587.

93. **Lee, J. Y., D. Hwang, and S. Gillam.** 1996. Dimerization of Rubella Virus Capsid Protein Is Not Required for Virus Particle Formation. Virology **216:**223-227.

94. **Lee, J.-Y., M. JA, and B. DS.** 1994. Characterization of rubella virus replication complexes using antibodies to double-stranded RNA. Virology **200:**307-312.

95. **Lee, J. Y., J. A. Marshall, and D. S. Bowden.** 1999. Localization of Rubella Virus Core Particles in Vero Cells. Virology **265:**110-9.

96. **Leiser, R., and P. Kaufmann.** 1994. Placental Strucure: in a comparative aspect. Experimental and Clinical Endocrinology **102:**122-134.

97. **Leung, D. N., S. C. Smith, K. F. To, D. S. Sahota, and P. N. Baker.** 2001. Increased placental apoptosis in pregnancies complicated by preeclampsia. Am J Obstet Gynecol **184:**1249-50.

98. **Li, P., D. Nijhawan, I. Budihardjo, S. M. Srinivasula, M. Ahmad, E. S. Alnemri, and X. Wang.** 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell **91:**479-89.

99. **Liang, Y., and S. Gillam.** 2000. Mutational Analysis of the Rubella Virus Nonstructural Polyprotein and Its Cleavage Products in Virus Replication and RNA Synthesis. Journal of Virology **74:**5133-41.

100. **Liu, X., J. Yang, A. M. Ghazi, and T. K. Frey.** 2000. Characterization of the zinc binding activity of the rubella virus nonstructural protease. J Virol **74:**5949-56.
101. **Liu, X., H. Zou, C. Slaughter, and X. Wang.** 1997. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell **89:**175-84.
102. **Liu, Z., D. Yang, Z. Qui, K.-T. Lim, P. Chong, and S. Gillam.** 1996. Identification of Domains in Rubella Virus Genomic RNA and Capsid Protein Necessary for Specific Interaction. Journal of Virology **70:**2184-2190.
103. **Lorenzo, H. K., S. A. Susin, J. Penninger, and G. Kroemer.** 1999. Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death Differ. **6:**516-24.
104. **Lotem, J., and L. Sachs.** 1996. Differential suppression by protease inhibitors and cytokines of apoptosis induced by wild-type p53 and cytotoxic agents. Proceedings of the National Academy of Science, USA **93:**12507-12512.
105. **MacCalman, C. D., E. E. Furth, A. Omigbodun, K. F. Kozarsky, C. Coutifaris, and J. F. r. Strauss.** 1996. Transduction of human trophoblast cells by recombinant adenoviruses is differentiation dependent. Biology of Reproduction **54:**682-91.
106. **MacLean, G. D., J. Seehafer, A. R. E. Shaw, M. W. Kieran, and B. M. Longenecker.** 1982. Antigenic heterogencity of human colorectal cancer lines analysed by a panel of monoclonal antibodies. I. Heterogenous expression of Ia-like and HLA-like antigenic determinants. Journal of the National Cancer Institute **69:**357-364.
107. **Magliano D, Marshall JA, Bowden DS, Vardaxis N, Meanger J, and L. JY.** 1998. Rubella virus replication complexes are virus-modified lysosomes. Virology **240:**57-63.
108. **Marr, L. D., C. Y. Wang, and T. K. Frey.** 1994. Expression of the rubella virus nonstructural protein ORF and demonstration of proteolytic processing. Virology **198:**586-92.
109. **Martinez, L. D., and M. T. Zapata.** 2002. Apoptosis induction by the infection with Gilchrist strain of rubella virus. J Clin Virol **25:**309-15.
110. **Mastromarino, P., L. Cioe, S. Rieti, and N. Orsi.** 1990. Role of membrane phospholipids and glycolipids in the Vero cell surface receptor for rubella virus. Medical Microbiology and Immunology **179:**105-114.
111. **Mastromarino, P., S. Rieti, L. Cioe, and N. Orsi.** 1989. Binding sites for rubella virus on erythrocyte membrane. Archives of Virology **107:**15-26.
112. **Megyeri, K., K. Berencsi, T. D. Halazonetis, G. C. Prendergast, G. Gri, S. A. Plotkin, G. Rovera, and E. Gonczol.** 1999. Involvement of p53-Dependent Pathway in RUbella Virus-Induced Apoptosis. Virology **259:**74-84.
113. **Morrish, D. W., J. Dakour, H. Li, J. Xiao, R. Miller, R. Sherburne, R. C. Berdan, and L. J. Guilbert.** 1997. In Vitro Cultured Human Term Cytotrophoblast: A Model for Normal Primary Epithelial Cells Demonstrating a Spontaneous Differentiation Programme that Requires EGF for Extensive Development of Syncytium. Placenta **18:**577-85.

114. **Nakhasi, H. L., D. Zheng, I. K. Hewlett, and T.-Y. Liu.** 1988. Rubella virus replication: effect of interferons and actinomycin D. Virus Research **10:**1-15.
115. **Nardone, A., N. J. Gay, and W. J. Edmunds.** 2002. Congenital rubella: down but not out. Lancet **360:**804.
116. **Nicholson, D. W., and N. A. Thornberry.** 1997. Caspases: killer proteases. Trends Biochem Sci. **22:**299-306.
117. **O'Connor, L., D. C. S. Huang, L. A. O'Reilly, and A. Strasser.** 2000. Apoptosis and cell division. Current Opinion in Cell Biology **12:**257-263.
118. **Oker-Blom, C., N. Kalkkinen, L. Kaariainen, and R. F. Pettersson.** 1983. Rubella Virus Contains One Capsid Protein and Three Envelope Glycoproteins, E1, E2a, and E2b. Journal of Virology **46:**964-973.
119. **Oker-Blom, C., I. Ulmanen, L. Kaariainen, and R. F. Pettersson.** 1984. Rubella Virus 40S Genome RNA Specifies a 24S Subgenomic mRNA That Codes for a Precursor to Structural Proteins. Journal of Virology **49:**403-8.
120. **Oltvai, Z. N., C. L. Milliman, and S. J. Korsmeyer.** 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell **74:**609-19.
121. **Ornoy, A., S. Segal, M. Nishmi, A. Simcha, and W. Z. Polishuk.** 1973. Fetal and placental pathology in gestational rubella. American Journal of Obstetrics and Gynecology **116:**949-956.
122. **Osborn, M., and K. Weber.** 1983. Tumor diagnosis by intermediate filament typing: A novel tool for surgical pathology. Laboratory Investigation **48:**372.
123. **Pardi, G., A. M. Marconi, and I. Cetin.** 2002. Placental-fetal interrelationship in IUGR fetuses--a review. Placenta **23 Suppl A:**S136-41.
124. **Payne, S. G., D. N. Brindley, and L. J. Guilbert.** 1999. Epidermal Growth Factor Inhibits Ceramide-Induced Apoptosis and Lowers Ceramide Levels in Primary Placental Trophoblasts. Journal of Cellular Physiology **180:**263-70.
125. **Petruzziello, R., N. Orsi, S. Macchia, S. Rieti, T. K. Frey, and P. Mastromarino.** 1996. Pathway of rubella virus infectious entry into Vero cells. J Gen Virol **77:**303-308.
126. **Prada, J. A., and R. C. Tsang.** 1998. Biological mechanisms of environmentally induced causes of IUGR. European Journal of Clinical Nutrition **52:**S21-S28.
127. **Pridjian, G., and J. B. Puschett.** 2002. Preeclampsia. Part 1: clinical and pathophysiologic considerations. Obstet Gynecol Surv. **57:**598-618.
128. **Pugachev, K. V., and T. K. Frey.** 1998. Effects of defined mutations in the 5' nontranslated region of rubella virus genomic RNA on virus viability and macromolecule synthesis. J Virol **72:**641-50.
129. **Pugachev, K. V., and T. K. Frey.** 1998. Rubella virus induces apoptosis in culture cells. Virology **250:**359-70.
130. **Redline, R. W., and C. R. Abramowsky.** 1985. Clinical and Pathologic Aspects of Recurrent Placental Villitis. Human Pathology **16:**727-31.
131. **Reef, S. E., T. K. Frey, K. Theall, E. Abernathy, C. L. Burnett, J. Icenogle, M. M. McCauley, and M. Wharton.** 2002. The Changing Epidemiology of Rubella in the 1990's On the Verge of Elimination and New Challenges for Control and Prevention. JAMA **287:**464-472.

132. **Roberts, J. M.** 2000. Preeclampsia: what we know and what we do not know. Semin Perinatol. **24:**24-8.
133. **Robertson, S. E., F. T. Cutts, R. Samuel, and J. L. Diaz-Ortega.** 1997. Control of rubella and congenital rubella syndrome (CRS) in developing countries, Part 2: vaccination against rubella. Bull World Health Organ. **75:**69-80.
134. **Rotonda, J., D. W. Nicholson, K. M. Fazil, M. Gallant, Y. Gareau, M. Labelle, E. P. Peterson, D. M. Rasper, R. Ruel, J. P. Vaillancourt, N. A. Thornberry, and J. W. Becker.** 1996. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat Struct Biol. **3:**619-25.
135. **Saidi, S., and K. Naficy.** 1969. Subcutaneous and Intranasal Administration of RA 27/3 Rubella Vaccine. American Journal of Diseases in Children **118:**209-212.
136. **Sakahira, H., M. Enari, and S. Nagata.** 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature **391:**96-9.
137. **Samuel, C. E.** 2001. Antiviral Actions of Interferons. Clinical Microbiology Reviews **14:**778-809.
138. **Schwartz, D. A., and A. J. Nahmias.** 1991. Human Immunodeficiency Virus and the Placenta. Annals of Clinical and Laboratory Science **21:**264-74.
139. **Schwartzman, R. A., and J. A. Cidlowski.** 1993. Apoptosis: The Biochemistry and Molecular Biology of Programmed Cell Death. Endocrine Reviews **14:**133-151.
140. **Sedwick, W. D., and F. Sokol.** 1970. Nucleic Acid of Rubella Virus and Its Replication in Hamster Kidney Cells. J Virol **5:**478-89.
141. **Selzer, G.** 1963. Virus Isolation, Inclusion Bodies, and Chromosomes in a Rubella-Infected Human Embryo. The Lancet:336-7.
142. **Slee, E. A., H. Zhu, S. C. Chow, M. MacFarlane, D. Nicholson, W., and G. M. Cohen.** 1996. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK) inhibits apoptosis by blocking the processing of CPP32. Biochem. J. **315:**21-24.
143. **Smith, S. C., P. N. Baker, and E. M. Symonds.** 1997. Increased placental apoptosis in intrauterine growth restriction. American Journal of Obstetrics and Gynecology **177:**1395-1401.
144. **Smith, S. C., P. N. Baker, and E. M. Symonds.** 1997. Placental apoptosis in normal human pregnancy. American Journal of Obstetrics and Gynecology **177:**57-65.
145. **Stanwick, T. L., and J. V. Hallum.** 1974. Role of Interferon in Six Cell Lines Persistently Infected with Rubella Virus. Infection and Immunity **10:**810-815.
146. **Strasser, A., L. O'Connor, and V. M. Dixit.** 2000. Apoptosis Signaling. Annual Reviews in Biochemistry **69:**217-45.
147. **Tam, T. W. S.** 1998. Following up on unfinished business -prenatal rubella screening and postpartum vaccination. CMAJ **159:**1117-8.
148. **Teodoro, J. G., and P. E. Branton.** 1997. Regulation of Apoptosis by Viral Gene Products. J Virol **71:**1739-1746.
149. **Thornberry, N. A., and Y. Lazebnik.** 1998. Caspases: enemies within. Science **281:**1312-6.

150. **Tondury, G., and D. W. Smith.** 1966. Fetal rubella pathology. The Journal of Pediatrics **68:**867-879.
151. **United-States** 1994, posting date. Rubella and COngenital Rubella Syndrome - United States January 1, 1991-May 7, 1994. Epidemiology Program Office - Centre for Disease Control Volume 43(21);391-401. [Online.]
152. **United-States** 1997, posting date. Rubella and Congenital Rubella Syndrome - United States, 1994-1997. Epidemiology Program Office - Center for Disease Control Volume 46(16);350-4. [Online.]
153. **Vandenbosche, R. C., and J. T. Kirchner.** 1998. Intrauterine growth retardation. Am Fam Physician **58:**1384-90, 1393-4.
154. **Vaux, D. L., and A. Strasser.** 1996. The molecular biology of aopotosis. Proceedings of the National Academy of Science, USA **93:**2239-44.
155. **von Dadelszen, P., and L. A. Magee.** 2002. Could an infectious trigger explain the differential maternal response to the shared placental pathology of preeclampsia and normotensive intrauterine growth restriction? Acta Obstet Gynecol Scand. **81:**642-8.
156. **Walker, N. P., R. V. Talanian, K. D. Brady, L. C. Dang, N. J. Bump, C. R. Ferenz, S. Franklin, T. Ghayur, M. C. Hackett, L. D. Hammill, and e. al.** 1994. Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)2 homodimer. Cell **78:**343-52.
157. **Waxham, M. N., and J. S. Wolinsky.** 1983. Immunochemical identification of rubella virus hemagglutinin. Virology **126:**194-203.
158. **Webster, W. S.** 1998. Teratogen Update: Congenital Rubella. Teratology **58:**13-23.
159. **White, D. O., and F. J. Fenner.** 1994. Medical Virology. Academic Press Limited.
160. **Wilson, K. P., J. A. Black, J. A. Thomson, E. E. Kim, J. P. Griffith, M. A. Navia, M. A. Murcko, S. P. Chambers, R. A. Aldape, S. A. Raybuck, and e. al.** 1994. Structure and mechanism of interleukin-1 beta converting enzyme. Nature **370:**270-75.
161. **Wilson, M. L., T. M. Goodwin, V. L. Pan, and S. A. Ingles.** 2003. Molecular epidemiology of preeclampsia. Obstet Gynecol Surv **58:**39-66.
162. **Wong, K. T., W. S. Robinson, and T. C. Merigan.** 1971. Inhibition of Rubella Virus-Specific RNA Synthesis by Interferon. P. S. E. B. M. **136:**615-8.
163. **Yao, J., and S. Gillam.** 2000. A Single-Amino-Acid Substitution of a Tyrosine Residue in the Rubella Virus E1 Cytoplasmic Domain Blocks Viirus Release. Journal of Virology **74:**3029-36.
164. **Yui, J., M. I. Garcia-Lloret, A. J. Brown, R. C. Berdan, D. W. Morrish, T. G. Wegmann, and L. J. Guilbert.** 1994a. Functional, Long-term Cultures of Human Term Trophoblasts Purified by Column-elimination of CD9 Expressing Cells. Placenta **15:**231-46.
165. **Yui, J., M. I. Garcia-Lloret, T. G. Wegmann, and L. J. Guilbert.** 1994b. Cytotoxicity of Tumour Necrosis Factor-alpha and Gamma-interferon Against Primary Human Placental Trophoblasts. Placenta **15:**819-35.
166. **Zimmermann, K. C., and D. R. Green.** 2001. How cells die: Apoptosis pathways. J Allergy Clin Immunol **108:**S99-103.